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14. ABSTRACT

Bioterrorism preparedness for infectious disease (BTPID), as part of homeland defense initiatives, continues to advance. Significant opportunities exist for new research and development of bioinformatics and telecommunications solutions for BTPID that can complement ongoing initiatives. With this in mind, this funded effort, with additional proposal modifications, progressed from a planning study, identification of problems and potential solutions, analyses of potential solutions, all resulting in important recommendations and knowledge. This project also helped to establish a Joint Clinical Research Center in Thailand. In addition to the effort described above, an additional modification was added to the existing proposal to expand relationships with nations in Southeast Asia (SEA) which will help foster bioterrorism related infectious disease research in Thailand and SEA in general. This modification enabled advanced simulation-based training on-site during the 2005 APMMC held in Vietnam. The project in its entirety represents a strengthening of knowledge and resources related to bioterrorism and infectious disease as well as a strengthening in Asia-Pacific relationships that are beneficial in working future problems related to bioterrorism, infectious disease outbreaks, and effective exchange of information. Publications resulting from certain efforts in this project, also document this knowledge for application wherever it may be needed in the future. This final report summarizes the spectrum of objectives, accomplishments, and products from work conducted over the entire research period.

15. SUBJECT TERMS
broadband medical networking, bioterrorism preparedness, bioinformatics, telecommunications, clinical informatics, clinical trials, emerging infectious diseases, simulation training

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Introduction

Bioterrorism preparedness for infectious disease (BTPID), as part of homeland defense initiatives, continues to advance. Significant opportunities exist for new research and development of bioinformatics and telecommunications solutions for BTPID that can complement ongoing initiatives. With this in mind, this funded effort, with additional proposal modifications, progressed from a planning study to implementation of a research and surveillance network. Initially, through meetings with key stakeholders, there was an identification of problems and analyses of potential solutions, all resulting in important recommendations and knowledge. As a result, this project also helped to establish a Joint Clinical Research Center in Thailand, which is serving as a focal point for future infectious disease research and bio-surveillance. In addition to the effort described above, an additional modification was added to the existing proposal to expand relationships with nations in Southeast Asia (SEA) which will help foster bioterrorism related infectious disease research in Thailand and SEA in general. This modification enabled advanced simulation-based training on-site during the 2005 APMMC held in Vietnam. The project in its entirety represents a strengthening of knowledge and resources related to bioterrorism and infectious disease as well as a strengthening in Asia-Pacific relationships that are beneficial in working future problems related to bioterrorism, infectious disease outbreaks, and effective exchange of information. The published works resulting from certain efforts in this project, also document this knowledge for application and use wherever it may be needed in the future.

This final report will summarize the spectrum of objectives, accomplishments, and resulting products, representing the work conducted over the entire research period.

The accomplishments of this project are associated with each task in the Statement of Work. The tasks from the Statement of Work for each year are as follows:

Year 1:

The main objective for the first year funding was to identify problems and proposed alternative bioinformatics and telecommunications solutions for (a) BTPID surveillance for both military and civilian populations emphasizing the use of secure, redundant, real-time networks and (b) BTPID outbreak management. This was accomplished in two steps: Year 1 focused on data gathering in preparation for year 2 which focused on demonstration, analysis, and recommendations. Year 1 work was accomplished through the following tasks:

1. Hold regular working meetings and retreats done in person or through teleconferencing.
2. Host a summit at a period three-fourths through the year 1 grant cycle to gather national and international experts in the field. This served as information gathering for year 2 analysis and recommendations for BTPID.

3. Define the problems and accompanying solutions for a wide variety of potential scenarios such as Dengue Fever (2001 Hawaii outbreak), Anthrax, and Smallpox epidemics.
4. Demonstrate solutions and evaluate potential solutions.

Year 2:

Year 2 focused on demonstration, analysis, and recommendations for BTPID surveillance. This was accomplished through the following tasks:

1. Define bioterrorism problem areas
2. Identify needs associated with the above task
3. Provide recommendations for future research

This particular grant year included three modifications to the BT grant (see next section).

Year 3:

Year 3 helped support the establishment of (1) a Joint Clinical Research Center (JCRC) in Thailand and (2) Advanced Simulation Based Training for the Asia Pacific Military Medicine Conference (APMMC). The tasks for the first objective were as follows:

1. Identify locations and partners for forming a Clinical Trials Center in emerging infectious diseases
2. Outline the structure of partnerships and funding from collaborating organizations for a Clinical Trials Center
3. Provide seed funding to the initiative to fund infrastructure and administrative support
4. Develop alternative and traditional funding sources through government and private industry

Part-time administrative support with both nursing and physician coverage of the JCRC was instituted. These individuals were also hired to support the HIV trials that have formed the nucleus of clinical research at the JCRC.

For the second objective an additional modification was added to the existing proposal to expand relationships with nations in Southeast Asia (SEA) which will help foster bioterrorism related infectious disease research in Thailand and SEA in general. UH has a growing relationship with the Asia Pacific Military Medical Conference (as attendees and presenters). The APMMC conference assembles all SEA military medical officers for one week annually and provides an outstanding opportunity to bring advanced simulation based training to the attendees (while also providing a more significant presence for UH in SEA). The aim of this additional effort was to provide two days of didactic sessions followed by a day-long training session for SEA medical officers and bring the conference to a new level. The team delivering the program to the conference consisted medical leaders of the Winter Institute for Simulation, Education and Research

(WISER) of the University of Pittsburgh Medical Center and UH. The ultimate goal in the future is to become an annual event at APMMC where UH can develop stronger associations throughout SEA, thus making it more capable of expanding its infectious disease research interests in the region.

With this in mind, the objectives of this grant modification was (a) for UH to partner with the Winter Institute for Simulation, Education and Research (WISER) of the University of Pittsburgh Medical Center to combine its on-line/live training curriculum with the simulation training from the University of Hawaii (UH) to develop an advanced simulation-based training for the APMMC and (b) to conduct the advanced simulation-based training on-site during the 2005 APMMC held in Vietnam. For the APMMC effort the tasks were:

1. Combine the WISER on-line/live training curriculum with the simulation training from UH Telehealth Institute to develop an advanced simulation-based training event for the APMMC
2. Conduct the advanced simulation-based training during the APMMC held in Vietnam

Year 4:

Year 4 continued with development of the JCRC. Additional grants were formulated and submitted through the HIV group. In one study, identification of a cohort at the earliest time of infection was approved through the DOD IRB at AFRIMS and its Command. Some funds from our grant were used to purchase kits for this study. In addition, other funding continued for HIV clinical research, and the part-time administrative support for the physician director was eliminated as his research duties increased, again speaking for success of the JCRC.

For support of simulation training for the APMMC, funding from another source was obtained to support the program. This included meeting support from TATRC and private industry support from a simulation leader, Laerdal.

Training support was provided to a Thai investigator to attend a training course in the US, which will assist the UH investigators while building research capability for our Thai counterparts.

Body

The following is a description of the research accomplishments for the efforts associated with each year of this award:

Year 1:

The first year of this effort provided a valuable foundation of knowledge and access to nationwide experts that were instrumental for the analyses that followed in year 2. The timeliness of this effort is important to underscore, as the US was preparing its infrastructure to deal with a future threat of bioterrorism. A Bioterrorism Retreat in Maui and a Bioterrorism Summit in Honolulu resulted in information that is an important archive of current knowledge applicable to BT response needs. It is important to make the information available through an open source for all interested individuals. We had succeeded in doing this through the TATRC website.

Key Research Accomplishments

- Establishment of core group of experts for future discussion/analysis of compiled information regarding problems/solutions for bioterrorism preparedness
- Establishment of Pacific Western Alliance for Biomedical Intelligence (PACWEST-ABI) – a consortium to address unsolved issues of BTPID.
- Completion of successful Retreat and Summit, bringing together national and international experts in a variety of fields and applying their knowledge and experience to bioterrorism preparedness problems
- Compilation and archiving of up-to-date information regarding BT issues and possible solutions
- Establishment of BT Series web pages on the TATRC website.

Year 2:

The information acquired during year 1 was archived and is available through the TATRC website, together with the analyses and recommendations from the year 2 portion of this project. In separate modifications to the original cooperative agreement, a report was prepared for DTRA and their review to assist in chemical and biological preparedness, and funding was initiated for a clinical research center in Thailand. Lastly, a travel modification was provided to support the Asia-Pacific Military Medical Conference in Hanoi, Vietnam in May 2005.

The following is a description of the research accomplishments associated with the tasks in the Statement of Work.

Key Research Accomplishments

- • The Dengue outbreak in Hawaii and its synopsis was provided as part of the year 2 annual report. It provides an interesting look at an infectious disease outbreak that occurred synchronously with 9/11, thereby causing difficulty in managing the outbreak due to disruption of airline flights and processing of specimens from a national laboratory in CONUS US. As such, this report provides valuable insight for the development of local and regional initiatives in preparation for bioterrorism acts. Dengue can also serve as a model for vector

borne illnesses. This report was provided with the Yr. 2 Annual Report but has since been update and modified for publication and is currently in press.

- • A meeting was conducted in June 2004 exploring the possibility of developing a Joint Clinical Research Center (JCRC) in Infectious Disease in Thailand. This provided an important look at the possibility of establishing such a Center, where clinical research can be readily conducted on patient populations with infectious diseases, that are listed as potential bioterrorism agents by the CDC.

- • Proposal Modification #1: Based on the June meeting in Bangkok, the grant was modified with additional funding to assist with the development of a Joint Clinical Research Center in association with the Armed Forces Research Institute of Medical Sciences (AFRIMS), Phramgkutlao Medical Center (PMK) of the Royal Thai Army, and the University of Hawaii (UH). Initial results were positive, with UH grants in HIV being the first to run through the Center. The grant provided for infrastructure or for pilot projects.

- • Proposal Modification #2: An education and training modification to the current grant was also provided to assist with the further development of the JCRC. Providing education and training activities is a natural extension of TATRC and this initiative, providing important inroads into relationships being developed with potential infectious disease research partners in Southeast Asia. The major initiative supported through this modification was the Asia Pacific Military Medical Conference (APMMC) that is primarily funded through US Army Pacific. In conjunction with WISER, the University of Hawaii provided training to military medical officers at this annual meeting in May 2005 in Hanoi.

- • Proposal Modification #3: The final positive development for year 2 was modification of the grant to provide support to the Defense Threat Reduction Agency (DTRA). With funding provided through DTRA, a UH individual was hired for this program. UH provided valuable short-term support to DTRA in providing a report to assist DTRA in defining goals, developing interagency agreements, and progressing rapidly in preparation against chemical and biological warfare.

Year 3:

The Yr. 3 effort helped support the establishment of the Joint Clinical Research Center in Thailand and simulation training to the APMMC. The following is a list of key research accomplishments emanating from this effort:

<u>Key Research Accomplishments</u>

- The University of Hawaii provided support toward the establishment of a Joint Clinical Research Center (JCRC) in Thailand by funding a 10% FTE UH faculty member (George Watt, M.D.), who provided important administrative time to the JCRC. In addition, a part-time nurse coordinator was hired to assist with Center administration.

- The University of Hawaii's HIV working group began the initiation of a clinical trial (NeuroAIDS Study 001) in conjunction with the Center (a cross-sectional NeuroAIDS protocol that was designed as a joint collaborative attempt between researchers at the University of Hawaii, Phramongkutlao (PMK) Hospital and AFRIMS). For assigned time from this grant, both Dr. Watt and the nurse coordinator did not conduct research.

- A new partnership with HIVNAT (HIV Netherlands Australia Thailand Research Collaboration) began, operating under the name SEARCH. A SEARCH website has been added under the Hawaii AIDS Clinical Research Program website: http://www.hawaii.edu/hacrp/search.htm. SEARCH partners include PMK, U.S. AFRIMS, HIVNAT and UH. The SEARCH research/training international infrastructure has lead to the following six grant proposals/projects.

(Funded) **Macrophages, HAART, And HIV-1 dementia in Thailand**. (1 R21 MH072388-01) V. Valcour PI **Funded, July 2004**
This awarded funding supports the longitudinal aspects of the original NeuroAIDS study; $300,000 total direct funding over 2 years.

(Funded) **International HIV/AIDS Training Center** (Gilead Pharmaceutical)
This training center will be designed to train SE Asia civilian physicians in HIV/AIDS care and management. Initial group of trainees is scheduled for early 2006. Funded for total cost of $100,000.

(Funded) **President's Emergency Plan for AIDS Relief (PEPFAR)** (via Department of Defense CoE)
Funding will support training of 8-12 Vietnamese physicians in HIV care and management/year. Direct costs of approximately $210,000/ year x 5 years.

(Pending) **Safety, Tolerability and Immunogenicity of ACAM3000 Modified Vaccinia Ankara (MVA) Small Pox Vaccine in HIV-Seropositive Subjects who are Vaccinia Naïve** (Acambis Pharmaceuticals) Review of proposal for SEARCH to open in Bangkok pending. Direct costs of approximately $200,000 total for study.

(Awarded) **HIV/AIDS Regional Training and Research Center in Asia** (Hui)
The funds support the University of Hawaii efforts to develop training and research infrastructure in HIV in Asia. Direct costs of approximately $300,000 x one year.

The Hawaii AIDS Clinical Trials Unit (HACTU) has been established as part of the AIDS Clinical Trial Network. This effort includes a laboratory and consultation center at

Leahi Hospital which produces research in the neurological and metabolic aspects of HIV with a keen interest in the Native Hawaiian as well as Asian-Pacific Islander aspects. Clinical trials have been attracted to the unit from industry and a clinical advisory board has been assembled that informs local practitioners of what research is being done. The personnel of this unit are significant contributors to national and international studies and have shed new light on the disease through their various publications (see Appendices 1 through 4).

- UH combined efforts with the WISER Institute at the University of Pittsburgh Medical Center to use its online/live training curriculum with the simulation-based training already existing at UH to develop an advanced simulation-based training event (Simulation Symposium) for the Asia Pacific Military Medicine Conference (APMMC) in Vietnam. The APMMC is a US Army Pacific (USARPAC) program conducted in support of the Pacific Command (PACOM) Theater Security Cooperation Plan (TSCP). The training symposium was successfully accomplished during the conference.

Year 4:

The objectives of year 4 were to increase research and collaboration between the US and SEA and establish crucial foundations to more effectively deal with bioterrorism or infectious diseases in the future.

Building on the established relationships between UH, PMK, and AFRIMS, this grant helped to provide supplemental funds to assist in forming a Clinical Trials Center in emerging infectious diseases at PMK. The location is unparalleled for the study of emerging infectious diseases. UH and PMK have grown closer together through its collaborative grants, and AFRIMS and PMK have been long-time collaborators. A UH association will also readily permit NIH funded studies to be conducted at the Center.

Although based on foreign soil, such a Center has distinct advantages, as it is being forged in a new era of advanced computing and telecommunications. The existing Internet2-type link provides videoteleconferencing capability between investigators in Hawaii and Thailand, as well as other consultants in the US. This link also permits transfer of data to the UH's supercomputer for genomics or proteomics studies. The broadband linkage permits this Center to have a wealth of connectivity and computing power, but be located in the locale where patients are afflicted with the diseases of interest and can be studied more readily. The Center will provide a vehicle for the study of infectious diseases with full patient, laboratory (including basic science), and animal facilities. The current partnerships provide a unique opportunity to form this Center. TATRC's funding has been essential to provide administrative and infrastructure support to help stand up the Center as a collaborative entity

The following are the key accomplishments from the Yr. 4 effort:

<u>Key Research Accomplishments</u>

- **Preliminary Study of Early Primary HIV Infection in High Risk cohort** (Jerome Kim, LTC, MC). This study proposes to detect early primary HIV infection by pooled, ultrasensitive nucleic acid testing (NAT) in HIV sero-negative, high-risk populations using the Roche Amplicor 1.5 assay. In addition, the study proposes to determine viral genotypes from HIV negative NAT positive sera. Sera will be obtained from the Thai Red Cross AIDS Research Centers (TRCARC) Anonymous testing clinic. This study was approved by the DOD-AFRIMS Scientific Review Committee on 19 May 2006. The University of Hawaii (UH) Committee on Human Studies deemed this protocol as exempt on 1 August 2006. The Chulalongkorn University (CU) School of Medicine, Institutional Review Board (IRB) approved this study on 28 June 2006.

- (Funded) **International HIV/AIDS Training Center** (Gilead Pharmaceutical).This training center will be designed to train SE Asia civilian physicians in HIV/AIDS care and management. Initial group of trainees is scheduled for early 2006. Funded for total cost of $100,000.

- (Funded) **President's Emergency Plan for AIDS Relief (PEPFAR)** (via Department of Defense CoE). Funding will support training of 8-12 Vietnamese physicians in HIV care and management/year. Direct costs of approximately $210,000/ year x 5 years.

- This effort assists Vietnam with antiretroviral treatment programs and trains Vietnamese military physicians at Leahi Hospital.

- (Pending) **Safety, Tolerability and Immunogenicity of ACAM3000 Modified Vaccinia Ankara (MVA) Small Pox Vaccine in HIV-Seropositive Subjects who are Vaccinia Naïve** (Acambis Pharmaceuticals) Review of proposal for SEARCH to open in Bangkok pending. Direct costs of approximately $200,000 total for study.

- (Awarded) **HIV/AIDS Regional Training and Research Center in Asia** (Hui).The funds support the University of Hawaii efforts to develop training and research infrastructure in HIV in Asia. Direct costs of approximately $300,000 x one year.

- Supported training of Thai investigator, to improve research capabilities of the resident Thai investigators.

- Obtained outside funding through TATRC and private industry to support the APMMC simulation training that was funding through the proposal the previous year.

Reportable Outcomes

Year 1

Abstracts from the Maui Retreat, February 8 – March 2, 2003 (see http://www.tatrc.org – select "past meetings" the select "Bioterrorism Meeting Series Archive.")

Abstracts from Bioterrorism Summit, October 19-21, 2003 (see http://www.tatrc.org – select "past meetings" the select "Bioterrorism Meeting Series Archive.")

Presentations from Maui Retreat and Bioterrorism Summit (for presentation slides see http://www.tatrc.org – select "past meetings" the select "Bioterrorism Meeting Series Archive.")

Databases/Websites

Web pages have been created to showcase and archive the activities of the Bioterrorism Preparedness retreats and meetings. The web pages can be found at http://www.tatrc.org under the "Bioterrorism Meeting Series." In addition, information about the PACWEST ABI can be found on the http://www.tri.jabsom.hawaii.edu/tri (select "information" then PACWEST ABI".

Year 2

Dengue Fever Outbreak Hawaii, 2001 – A Bioterrorism Model for Vector Borne Illnesses (report submitted with Year 2 Annual Report). An updated modified version is now in press to appear as the following publication: The Dengue Fever outbreak in Hawaii: A bioterrorism model for vector-borne illnesses. International Journal of Healthcare Technology and Management (in press). (See attached final proof, Appendix 5).

Agenda for the Bioterrorism Preparedness Summit that took place from 15 June 2004 to 18 June 2004 in Bangkok, Thailand (previously submitted in Yr. 2 annual report).

Year 3

UH-PMK NeuroAIDS Study 001: Predictors of Neuro-cognitive decline and survival in HIV-infected Subjects (A Pilot Study). Report previously submitted as attachment in Year 3 Annual Report.

Agenda, abstract, and after action report for the Simulation Training, Trauma Life Support activity for the APMMC, Hanoi (May 9-13, 2005). These documents were previously submitted as attachment in Year 3 Annual Report.

Year 4

Two papers were submitted for publication during this period:

Garshnek, V. and Burgess, L. The Dengue Fever outbreak in Hawaii: A bioterrorism model for vector-borne illnesses. International Journal of Healthcare Technology and Management (in press). (See Appendix 5)

Garshnek, V. Infectious Disease Surveillance – A Review of Current Systems. In: Bio-event Disasters and the Public Health. Springer: New York (in press). (See Appendix 6)

Studies:

Approval documentation for study: "Preliminary Study of Early Primary HIV Infection in High Risk Cohort". See approval letter for exempt human use protocol from the University of Hawaii and the Dept. of the Army, Walter Reed Army Institute of Research (see Appendices 7 and 8). For study protocol see Appendix 9)

Publications from the Hawaii AIDS Clinical Trials Unit (also see Appendices 1 through 4)

Valcour, V., Yee, P., Williams, A.E., Shiramizu, B., Watters, M., Selnes, O., Paul, R., Shikuma, C., Sacktor, N. Lowest ever CD4 lymphocyte count (CD4 nadir) as a predictor of current cognitive and neurological status in human immunodeficiency virus type 1 infection – the Hawaii Aging with HIV Cohort. J.Neurovirol., 12(5): 387-91, 2006.

Chow, DC, Wood, R, Grandinetti, A., Shikuma, C., Schatz, I., Low, P. Cardiovagal autonomic dysfunction in relation to HIV-associated lipodystrophy. HIV Clin. Trials, 7(1): 16-23, 2006.

Yu, Q., Jones, B., Hu, N., Chang, H., Ahmad, S., Liu, J., Parrington, M., Ostrowski, M. Comparative analysis of tropism between canarypox (ALVAC) and vaccinia viruses reveals a more restricted and preferential tropism of ALVAC for human cells of the monocytic lineage. Vaccine, 24(40-41): 6376-91, Epub 2006.

Valcour, V., Paul, R. HIV infection and dementia in older adults. Clin Infect. Dis., 42(10): 1449-54, Epub 2006.

Conclusion

The first year of this effort was successfully completed and provided a valuable foundation of knowledge and access to nationwide experts that were instrumental for the analyses that followed in year 2. The BT preparedness material presented during the Retreat and Summit emphasized the need to fully understand various infectious diseases in their various aspects of transmission, illness profile and time-course, and methods used to contain their spread and destructive consequences. The various tools available to mobilize for BT preparedness are impressive – supercomputers, biosensors, modeling, etc. The expertise and technology exist and it is encouraging to know that the experts are able to readily apply their knowledge and skills to BT preparedness issues. These experts

are the workforce that should actively participate in discussions of the gaps and needs in BT preparedness that need a timely solution. In many cases, existing projects can be modified to address BT preparedness needs.

The information presented during the Bioterrorism retreat and Summit is an important archive of knowledge applicable to BT response needs. It is important to make the information available through an open source for all interested individuals to use to address BT issues. We have succeeded in doing this through the TATRC website and hope to bring a wider audience together to experience the information and contact the presenters for additional insight.

The second year of this effort was successfully completed and provides a valuable foundation of knowledge, access to expertise and potential solutions. The second year focused on analysis and potential solution (supplemented by additional literature reviews and meetings as required) to yield recommendations that could be utilized as part of a homeland defense initiative and be applicable as part of a force protection initiative for garrisoned or deployed DOD units, who are highly visible targets for terrorist or conventional biological warfare threats. A multidisciplinary team conducted this study and consisted of members from the DOD, State of Hawaii Department of Health, Maui High Performance Computing enter (MHPCC), University of Hawaii(Telemedicine, Environmental Health, Bioinformatics, Infectious Disease), University of Southern California (Image Processing and Informatics Lab) and Stanford University-NASA Ames (National Biocomputation Center). In separate modification to the original cooperative agreement, a report was prepared for DTRA for their review to assist in chemical and biological preparedness, and funding was initiated for a clinical research center in Thailand. Also a travel modification was provided to support the Asia-Pacific Military Medical Conference in Hanoi, Vietnam in May 2005.

Building on the established relationships between UH, PMK, and AFRIMS, the third year of this grant helped to provide supplemental funds to assist in forming a Clinical Trials Center in emerging infectious diseases at PMK. The location is unparalleled for the study of emerging infectious diseases. UH and PMK have grown closer together through its collaborative grants, and AFRIMS and PMK have been long-time collaborators. A UH association will also readily permit NIH funded studies to be conducted at the Center.

Although based on foreign soil, such a Center has distinct advantages, as it is being forged in a new era of advanced computing and telecommunications. The existing Internet2-type link provides videoteleconferencing capability between investigators in Hawaii and Thailand, as well as other consultants in the US. This link also permits transfer of data to the UH's supercomputer for genomics or proteomics studies. The broadband linkage permits this Center to have a wealth of connectivity and computing power, but be located in the locale where patients are afflicted with the diseases of interest and can be studied more readily. The Center will provide a vehicle for the study of infectious diseases with full patient, laboratory (including basic science), and animal facilities. The current partnerships provide a unique opportunity to form this Center.

TATRC's funding has been essential to provide administrative and infrastructure support to help stand up the Center as a collaborative entity

The opportunity through the Asia Pacific Military Medical Conference provided an outstanding method to bring advanced simulation based training to the SEA, while also providing a more significant presence for the University of Hawaii in SEA. Significant interest in development and/or enhancement of simulator training capacity was expressed by senior medical officers from Australia, Singapore, Vietnam, India, and Thailand. In addition, senior medical leaders in the Asia Pacific region expressed views that medical simulation training is a method with potential for integration with existing regional military training programs. Simulation training represents a novel method of transcultural medical education that should be further explored and expanded. We hope that this will become an annual event at APMMC, which will enable UH to develop stronger associations throughout SEA, thus making it more capable of expanding its infectious disease research interests in the region.

The fourth year of this effort was successfully completed. Through this effort important progress was made to increase and continue research and collaboration between the US and SEA and strengthen crucial foundations to more effectively deal with bioterrorism or infectious diseases in the future. Active research within the Joint Clinical Research Center in Thailand continues with approved studies and strong possibilities for further research and collaboration between the US and SEA. An effective Pacific bridge has been established with the aid of this project. It is our hope and intent that this bridge will enable a rapid and effective response in defending against infectious disease brought upon by Nature or engineered Bioterrorism events.

References

Bioterrorism Meeting Series. Available at http://www.tatrc.org

Continuing Guidance for Cooperative Agreement on Public Health Preparedness and Response for Bioterrorism Program Announcement 99051, May 2, 2003. Available at http://www.bt.cdc.gov/planning/continuationguidance/pdf/guidance_intro.pdf

PACWEST ABI. Available at http://www.tri.jabsom.hawaii.edu/tri/info-pacwestabi.php

Appendices

1. Lowest ever CD4 lymphocyte count (CD4 nadir) as a predictor of current cognitive and neurological status in human immunodeficiency virus type 1 infection – the Hawaii Aging with HIV Cohort.

2. Cardiovagal autonomic dysfunction in relation to HIV-associated lipodystrophy.

3. Comparative analysis of tropism between canarypox (ALVAC) and vaccinia viruses reveals a more restricted and preferential tropism of ALVAC for human cells of the monocytic lineage.

4. HIV infection and dementia in older adults

5. The Dengue Fever outbreak in Hawaii: A bioterrorism model for vector-borne illnesses.

6. Infectious Disease Surveillance – A Review of Current Systems

7. University of Hawaii Committee on Human Studies Letter

8. Dept. of the Army, Walter Reed Army Institute of Research Letter

9. Preliminary Study of Early Primary HIV Infection in High Risk cohort

APPENDIX 1.

Lowest ever CD4 lymphocyte count (CD4 nadir) as a predictor of current cognitive and neurological status in human immunodeficiency virus type 1 infection – the Hawaii Aging with HIV Cohort.

Lowest ever CD4 lymphocyte count (CD4 nadir) as a predictor of current cognitive and neurological status in human immunodeficiency virus type 1 infection - The Hawaii Aging with HIV Cohort

Authors: Victor Valcour [a]; Priscilla Yee [b]; Andrew E. Williams [a]; Bruce Shiramizu [a]; Michael Watters [a]; Ola Selnes [c]; Robert Paul [d]; Cecilia Shikuma [a]; Ned Sacktor [c]

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Abstract

Low CD4 lymphocyte count was a marker for neurological disease in human immunodeficiency virus type 1 (HIV-1); but is now less common among patients with access to highly active antiretroviral therapy. In this study, the authors determine the reliability of self-reported CD4 nadir and its predictive value for neurological status. The authors identify a high degree of reliability ($r = .90$). After adjusting for age, current CD4 count, and duration of HIV-1, CD4 nadir relates to a current diagnosis of HIV-associated dimentia (HAD) (odds ratio [OR]: 1.395 (1.106-1.761), $P = .005$) and distal symmetric polyneuropathy (DSPN) (OR: 1.479 (1.221-1.769, $P < .001$).

Keywords: AIDS dementia complex; CD4 lymphocyte count; polyneuropathies

APPENDIX 2.

Cardiovagal autonomic dysfunction in relation to HIV-associated lipodystrophy

unbound MEDLINE

Title Cardiovagal autonomic dysfunction in relation to HIV-associated lipodystrophy.

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Abstract **PURPOSE:** Human immunodeficiency virus (HIV)-associated lipodystrophy (LD) may be mediated by changes in the autonomic nervous system. We examined the autonomic function among HIV-infected patients with LD compared to HIV-infected patients without LD (non-LD) and HIV-negative controls (controls).
METHOD: This cross-sectional study examined cardiovagal autonomic function among the three groups. LD was defined in HIV-infected patients as increased visceral adipose accumulation and peripheral lipoatrophy. Cardiovagal autonomic testing was assessed by measuring heart rate variability during rest, paced breathing, and upright tilt and was analyzed in time and frequency domains.
RESULTS: Cardiovagal testing was performed on 58 participants: 26 controls, 20 non-LD, and 12 LD. After adjustment for visceral fat, time domain analysis showed decreased heart rate variability in patients with LD compared to the other groups ($p < .05$). The frequency domain analysis showed decreased high-frequency power and increased low- to high-frequency power ratio in the LD group compared to both groups during rest and to non-LD during tilt ($p < .05$).
CONCLUSION: Patients with LD have altered cardiovagal modulation. Patients with LD had lower heart rate variability and higher sympathetic modulation compared to non-LD and controls. These alterations may be prognostic of increased cardiovascular disease morbidity and mortality.

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APPENDIX 3.

Comparative analysis of tropism between canarypox (ALVAC) and vaccinia viruses reveals a more restricted and preferential tropism of ALVAC for human cells of the monocytic lineage

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Comparative analysis of tropism between canarypox (ALVAC) and vaccinia viruses reveals a more restricted and preferential tropism of ALVAC for human cells of the monocytic lineage

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Abstract

The poxviruses including canarypox (ALVAC) and vaccinia viruses are promising vaccine vectors in humans, but little is known about their biology in human cells. Using recombinant enhanced green fluorescence protein (EGFP)-expressing ALVAC and vaccinia viruses, we have focused here on a side-by-side comparison of ALVAC and vaccinia virus tropism for cells from human peripheral blood and bone marrow. Both ALVAC and vaccinia viruses showed a strong bias towards monocyte infection. ALVAC minimally infected $CD19^+$ B cells and was unable to infect *ex vivo* NK cells and T lymphocytes, whereas vaccinia virus could infect B lymphocytes and NK cell populations. Vaccinia virus was also able to infect T lymphocytes at low, but detectable levels that could be enhanced upon their activation. The observed preferential infection of ALVAC or vaccinia virus to monocytes was the result of preferential binding to this population, rather than lineage-specific differences in the expression of viral genes. Moreover, the level of CD14 expression on monocytes correlated with their preference to be infected with ALVAC or vaccinia virus. Both ALVAC and vaccinia viruses could infect immature monocyte derived dendritic cells (MDDCs), but only ALVAC infection induced their subsequent maturation. Vaccinia virus, however, showed greater tropism for mature MDDCs compared to ALVAC. Infection in human bone marrow cultures showed that ALVAC infection was restricted to a myelomonocytoid cell-specific $CD33^+$ cell population, while vaccinia virus showed a strong, but not exclusive, preference for these cells. These findings have implications in terms of choosing optimal pox virus derived vectors as vaccines in terms of reducing clinical reactogenicity and inducing dendritic cell (DC) maturation.

Keywords: Virus; ALVAC; Vaccinia; Monocyte; Dendritic cell; Myeloid cell

1. Introduction

Members of the pox virus family (*Poxviridae*) have in recent years, received considerable attention for the development of vaccine vectors that can induce humoral and cellular immunity against virus infections as well as immunotherapy for cancer [1–3]. Several advantages of these vectors for vaccine development include strict cytoplasmic replication

Abbreviations: DC, dendritic cell; EGFP, enhanced green fluorescence protein; MDDC, monocyte-derived DC; PBMC, peripheral blood mononuclear cell; SEB, staphylococcal enterotoxin B superantigen

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that is primarily abortive in human cells, and their good long term safety profile [4,5]. Canarypox (ALVAC) and vaccinia viruses are members of the *Chordopoxvirinae* subfamily of *Poxviridae* which comprises a large family of complex DNA viruses that replicate in the cytoplasm of vertebrate (*Chordopoxvirinae* subfamily or vertebrate poxviruses) and invertebrate (*Entomopoxvirinae* subfamily or insect poxviruses) cells. The most notorious member of *Chordopoxvirinae* subfamily, variola virus, causes smallpox and has claimed a greater number of human lives over the span of recorded history than all other infectious diseases combined [6]. Smallpox was finally eradicated in 1977, nearly two centuries after the introduction of prophylactic inoculations with vaccinia virus and cowpox. Vaccinia virus, the prototype member of the family *Poxviridae*, was used extensively in the past as the smallpox vaccine, and although was considered as a candidate vector for new recombinant vaccines, its application potential is limited by the pre-existence of vaccinia virus-immunity in the smallpox-vaccinated population as well as rare adverse affects ranging from eczema vaccinatum, to myocarditis, to vaccinial encephalitis, resulting in fatal complications and substantial morbidity in some individuals [7]. The highly attenuated canarypox virus strain, ALVAC, with a genome of approximately 300 kb pairs, has been extensively developed to express genes from rabies virus [5], canine distemper virus [8], feline leukemia virus [9], human immunodeficiency virus (HIV) [10–16], SIV [17,18], human cytomegalovirus [19], melanoma [20], and Japanese encephalitis virus [21]. Safe delivery of these antigens is insured by the inability of the avian host-restricted ALVAC virus to replicate in mammals. While human vaccines of most poxvirus vectors remain in experimental or clinical trial phases, ALVAC veterinary vaccines are now commercially available.

Understanding immunogenicity and reactogenicity of these vectors may also be obtained from studies of their effects on dendritic cells. For example, it has been determined that ALVAC infects immature myeloid derived dendritic cells (MDDCs) more easily than their mature counterpart, and this infection induces MDDC maturation as measured by marked upregulation of the maturation markers CD80, CD83, CD86, CD25, and DC-LAMP [22]. Maturation is driven by tumor necrosis factor alpha (TNF-α) secretion in response to ALVAC infection, as well as by the ingestion of cellular debris from apoptotic ALVAC-infected immature MDDCs [22]. A direct comparison with vaccinia virus has not been reported, although such information may be useful to understanding the comparable biological effects of these two virus vectors.

Surprisingly, despite extensive ongoing work in human clinical trials, there has until recently [23], been little information available regarding the range of infective tropism these virus vectors have in primary human cells. A recent paper surveyed the susceptibility of different leukocyte cell types in peripheral blood to vaccinia virus infection. Strong bias was shown towards the infection of peripheral monocytes, followed by B lymphocytes and NK cells. *Ex vivo* T lymphocytes were infected at low, but detectable levels [24]. Chahroudi et al. recently showed that activated T cells could also be easily infected with vaccinia virus [23]. Considering the substantial differences in host tropism, replicative capacity, and genome sequences between the vaccinia and canarypox viruses, it cannot be assumed that this specificity is paralleled by ALVAC. In addition to this consideration, the tropism for cells of similar lineage, *i.e.*, progenitors of leukocytes in bone marrow and monocyte-derived dendritic cells, has not yet been addressed for either vaccinia or canarypox virus. This knowledge is requisite for the rational development of an ALVAC vector and other poxvirus vectors in order to have enhanced immunogenicity and uncompromised safety. We have focused here on a side-by-side comparison of ALVAC and vaccinia virus capacities to infect leukocyte subsets in human bone marrow, peripheral blood, and monocyte-derived dendritic cells. We demonstrate that ALVAC and vaccinia virus exhibit infective cellular tropism for human myeloid lineage cell types, with a more restricted pattern of tropism for ALVAC. In addition, the tropism and effects of these two viruses on myeloid derived dendritic cells are compared and contrasted.

2. Materials and methods

2.1. Viruses and virus infection in vitro

Wild type ALVAC virus, a highly attenuated strain of canarypox virus, and recombinant ALVAC virus vCP1540 expressing enhanced green fluorescent protein (EGFP) were obtained from Sanofi-Pastuer (Toronto, Canada). A recombinant vaccinia virus expressing EGFP (EGFP-vaccinia) was a gift from Dr. Jonathan Yewdell (NIH, Bethesda, MD). EGFP-vaccinia was derived from a WR strain of vaccinia and has a fusion protein inserted consisting of the nucleoprotein of influenza A/Puerto Rico/8/34 fused to chicken OVA (257–264) epitope, followed by a COOH-terminal fusion to EGFP from aequorea Victoria [25]. Wild type ALVAC, vCP1540, and EGFP-vaccinia viruses were grown and titrated in primary chicken embryo fibroblasts (Charles River Laboratories, WI) in minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine.

Human peripheral blood mononuclear cells (PBMCs), monocytes, monocyte-derived dendritic cells (MDDCs), or bone marrow cells were infected at a multiplicity of infection (MOI) of 10 (10 viral particles per cell) with wild type ALVAC, recombinant ALVAC virus vCP1540, or EGFP-vaccinia virus. After 1 h adsorption at 37 °C, free virus particles were removed by washing twice with ice-chilled 2% FBS/PBS and cell pellets were resuspended in complete RPMI 1640 medium containing 10% FBS plus 2 mM glutamine, 25 mM HEPES, and antibiotics and cultured at 37 °C in a 5% CO_2 incubator for various time intervals.

2.2. Preparation of PBMCs, monocytes, monocyte-derived dendritic cells, and bone marrow cells

Heparinized human peripheral blood was obtained from healthy blood donors. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). For activation of T cells, PBMCs were activated with plate-bound anti-CD3/anti-CD28/anti-CD49d mAbs (all antibodies from Becton-Dickinson Pharmingen, incubated overnight at 1 μg/ml) or incubated with medium alone for 48 h at 37 °C. These stimulated PBMCs and unstimulated PBMCs isolated from the same donor were infected with ALVAC (vCP1540) or EGFP-vaccinia virus at an MOI of 10 for 16 h. Monocytes were separated from the PBMCs using multistep Percoll (Sigma, St. Louis, MO) gradient centrifugation, and then purified by depletion of contaminating B cells, T cells, NK cells, and granulocytes using antibody-conjugated magnetic beads in the Monocyte Negative Isolation Kit (Dynal, Oslo, Norway) according to the manufacturer's guidelines. The resulting cell preparations contained more than 95% monocytes, and <0.1% T and B lymphocytes, as assessed by CD14, CD4, CD8, and CD19 staining and flow cytometric analysis (data not shown). Monocyte-derived dendritic cells (MDDCs) were generated by a modification of a method previously described [26–28]. Briefly, purified peripheral monocytes were cultured at 1×10^6 cells/ml in medium consisting of RPMI 1640 plus 10% FBS, 2 mM glutamine, 25 mM HEPES, and antibiotics in the presence of 50 ng/ml recombinant human GM-CSF (GM-CSF) and 100 ng/ml recombinant human IL-4 (IL-4) (PeproTech, Rocky Hill, NJ). GM-CSF and IL-4 were added again on days 3 and 5 with the fresh complete RPMI 1640 medium. After 7 days of culture, more than 50% of the cells were $CD1a^{high}$, MHC class II^+, $CD80^{low}$, and $CD14^-$, which represents an immature DC (iDC) phenotype. The immature DCs were directly infected with vCP1540 or EGFP-vaccinia, or matured for 2–3 days by stimulation of soluble recombinant human CD40L trimer protein (CD40LT, a gift from Immunex, Seattle, WA) at a concentration of 2 μg/ml [27,28]. Human bone marrow (BM) aspirations were collected from the posterior iliac crest of three healthy donors, none of which had history of previous hematologic disorders, previous chemotherapy, or radiation therapy. BM mononuclear cells (BMMNC) were separated by Ficoll-Hypaque density gradient (1.077 g/ml) (Sigma Diagnostics, St. Louis, MO) and washed twice with Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY). Blood and BM samples were obtained according to guidelines established by Institutional Review Boards for Human Research at University of Toronto (Toronto, Canada).

2.3. Antibody, flow cytometric analysis, and fluorescence-activated cell sorting

The following anti-human monoclonal antibodies (mAbs) or polyclonal Abs conjugated with fluorochrome were purchased from BD PharMingen (San Diego, CA): anti-$CD1a^{FITC}$, anti-$CD3^{APC}$, anti-$CD4^{PerCP}$, anti-$CD8^{PE}$, anti-$CD10^{PE}$, anti-$CD14^{APC}$, anti-$CD19^{PE}$, anti-$CD33^{PerCP}$, anti-$CD56^{PE}$, anti-$CD69^{APC}$, anti-$CD80^{PE}$, anti-$CD83^{PE}$, anti-$CD86^{APC}$, anti-TNF-α^{APC}, anti-IL-12^{PE}, and isotype-matched control Abs conjugated with FITC, PE, PerCP, or APC. Purified monoclonal Abs of anti-human CD3, anti-human CD28, and anti-human CD49d were also purchased from BD PharMingen (San Diego, CA).

PBMCs, MDDCs, or bone marrow cells, infected or uninfected with vCP1540 or EGFP-vaccinia virus, were stained in PBS/1% FBS/0.02% NaN_3 using fluorochrome-conjugated Abs from BD PharMingen. Samples were subjected to flow cytometric analysis using a FACSCaliber (BD Biosciences, San Diego, CA) and the data were analyzed using FlowJo software (Tree Star, San Carlos, CA). Appropriate isotype controls were used at the same protein concentration as the test Ab and control staining was performed during every flow cytometric analysis. For intracellular staining, cells were permeabilized using Cytofix/Cytoperm Plus kit (BD PharMingen) according to the manufacturer's instructions. Intracellular staining was performed for detecting intracellular levels of TNF-α and IL-12 in DCs infected and uninfected with vCP1540 or vaccinia virus and INF-γ within activated $CD3^+$ T cells.

Fluorescence-activated cell sorting was performed used a FACStar Plus (Becton-Dickinson) equipped with 5 W argon and 30 mW helium neon lasers. PBMCs exposed or unexposed to wild type ALVAC or EGFP-vaccinia virus at 37 °C for 1 h were stained with a combination of mAbs of $CD3^{APC}$/$CD19^{PE}$ or $CD14^{APC}$/$CD56^{PE}$ and subjected to flow cytometric analysis. CD3, CD14, CD19, and CD56 fractions were sorted for DNA extraction.

2.4. DNA extraction, PCR, and dot blot hybridization

Cell fractions sorted by CD3, CD14, CD19, and CD56 markers from wild type ALVAC- or EGFP-vaccinia-exposed or -unexposed PBMCs were washed twice with ice-chilled PBS, and resuspended in 200 μl of PBS for DNA extraction using QIAamp DNA Blood Mini Kit according to the manufacturer's guidelines (QIAGEN). Purified DNA was quantified spectrophotopically and subjected to PCR or dot blot hybridization. A pair of oligonucleotides CNPV136F (5′-ATTGCGCGATGTAGATAAATGTTACAAAC-3′) and CNPV136R (5′-GCATCAAAGAGTATAGCTTCATACCCTG-3′) or VACV F4L.F389-409 (CGTTGGAAAACGTGAGTCCGG-3′) and VACV F4L.R774-754 (5′-ATTGGCGTTTTTGCAGCCAG-3′) were used as PCR primers to detect ALVAC and vaccinia virus sequences, respectively, in DNA extracted from $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, and $CD56^+$ NK cells sorted from ALVAC or vaccinia virus-exposed PBMCs. Amplification of the human house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a PCR control. The primers were GAPDH.For: 5′-CCTGGCCAAGGTCATCCATG-3′

and GAPDH.Rev: 5′-CTGCTTCACCACCTTCTTGA-3′. The human GAPDH gene consists of 9 exons and 8 introns with eukaryotic signals necessary for the transcription and translation of GAPDH mRNA. The oligonucleotides for GAPDH.For and GAPDH.Rev were derived from GAPDH exons 7 and 8, respectively, which flank 193 bp length of intron 7 [29]. 10 ng of DNA was subjected to PCR with PLATINUM *Taq* (Invitrogen, Inc.) according to the manufacturer's instruction. The PCR mixture containing 200 nM sense and antisense primers plus 2 U of PLATINUM *Taq* polymerase was denatured at 95 °C for 2 min, and then subjected to 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min. PCR products were size fractionated using ethidium-stained 1.5% agarose gel electrophoresis. Expected lengths of PCR amplicons were 525 bp, 386 bp, and 509 bp for ALVAC, vaccinia virus, and human GAPDH, respectively. Identities of PCR products were confirmed by sequencing.

One hundred nanograms of each DNA sample prepared from sorted PBMCs subsets were exposed or unexposed to ALVAC or vaccinia virus, diluted in 50 μl H_2O and then treated with an equal volume of 0.7N NaOH for 30 min on ice prior to addition of 100 μl of 2 M ammonium acetate. Duplicate 100 μl aliquots were dotted onto nylon membranes (Boehringer Mannheim) using the HYBRI-SLOT filtration manifold (Life Technologies, Gaithersburg, MD). Sample blots were washed twice with 200 μl 2 × SSC (0.3 M NaCl plus 0.03 M sodium citrate). The membranes were exposed to UV at 254 nm for 3 min for cross linking (Stratalinker, Stratagene, La Jolla, CA) and then hybridized with DIG-ddUTP-labeled ALVAC-specific probe CNPV136F (5′-ATTGCGCGATGTAGATAAATGTTACAAAC-3′), vaccinia-specific probe VACV F4L.F389-409 (CGTTGGAAAACGTGAGTCCGG-3′) or human house keeping gene probe GAPDH.Rev (5′-CTGCTTCACCACCTTCTTGA-3′) in PerfectHyb Plus Hybridization buffer (Sigma Chemical Co., St. Louis, MO) at 52 °C overnight. Membranes were then washed twice for 5 min each at 52 °C with 0.1% SSC containing 0.1% (w/v) SDS. Detection of the DIG-labeled nucleic acids with CSPD (Boehringer Mannheim) was performed according to the manufacturer's instructions. The hybridization signal was visualized by exposing membranes to Kodak XAR-5 film.

2.5. Statistical analysis

Data were compared using the Wilcoxon signed rank test for paired samples.

3. Results

3.1. Monocyte subsets within PBMCs are preferential targets for ALVAC or vaccinia virus infection

Recombinant vaccinia virus expressing green fluorescence protein (GFP-Vaccinia) was previously reported to show a strong bias towards peripheral monocyte (CD14 positive) infection. A much lesser degree of infection was observed in the B lymphocyte and NK cell populations. Resting T lymphocyte infection was only barely detectable by flow cytometric analysis [23,24]. This cellular infection tropism was consistently reproduced in the current study using a recombinant EGFP-vaccinia virus (Fig. 1A and B).

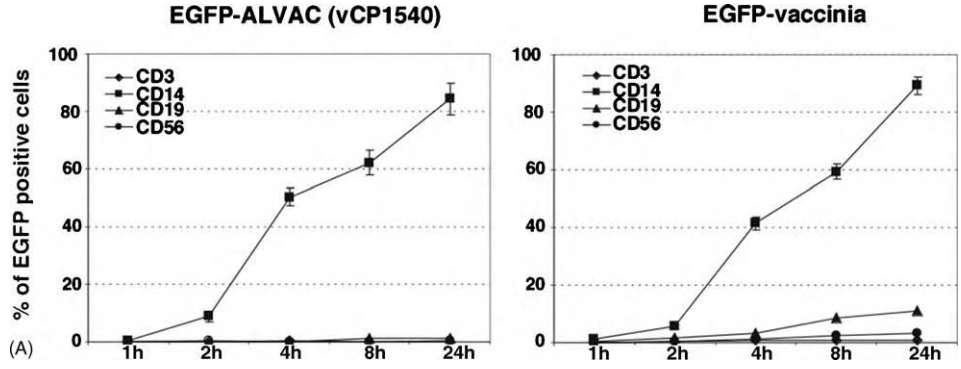

Fig. 1. Preferential infection of peripheral monocytes in PBMCs with ALVAC or vaccinia virus. Human PBMCs were exposed with an MOI of 10 of EGFP-expressing ALVAC recombinant virus vCP1540 or EGFP-vaccinia virus for 1 h at 37 °C in a 5% CO_2 incubator. Cells were washed twice with ice-chilled 2% FBS/PBS, and then cultured at various time intervals. The infected cells were stained with fluorochrome-conjugated Abs of $CD3^{APC}$/$CD4^{PerCP}$/$CD8^{PE}$, $CD14^{APC}$/$CD19^{PE}$, or $CD56^{PE}$, and then subjected to flow cytometric analysis. In (A) are kinetic data of ALVAC or vaccinia virus infection in PBMCs subsets from a healthy blood donor; a representative of six experiments is shown. *Y*-axis shows the percentage of EGFP-positive cells out of the total T cells (CD3), monocytes (CD14), B cells (CD19), or NK cells (CD56) as indicated. Infections are performed in triplicate and bars show standard error on the mean. In (B) one representative experiment shows the infection of T cells, B cells, monocytes, and NK cells with vCP1540 or EGFP-vaccinia virus at 4 h post-infection. Numbers in each plot indicates the percentage of EGFP-positive cells within the indicated PBMC subsets. In (C) are summary data of all experiments at 8 h post infection. In (D) activation of $CD3^+$ T cells, by plate bound anti-human CD3/CD28/CD49d mAbs, induced minor changes in permissiveness to infection by ALVAC but a modest enhancement of infection of T cells with vaccinia viruses. Representative data from one of three individuals are shown. Values in upper left quadrants indicate %EGFP-expressing cells of CD69 negative (unactivated) T cells and values in upper right quadrants indicate %EGFP-expressing cells of CD69 positive (activated) fraction. In (E) summary data of infection of CD69 negative (unactivated) and CD69 positive (activated) T cells by vCP1540 and vaccinia viruses from three experiments are shown. VACC represents vaccinia virus. Bars represent standard error on the mean.

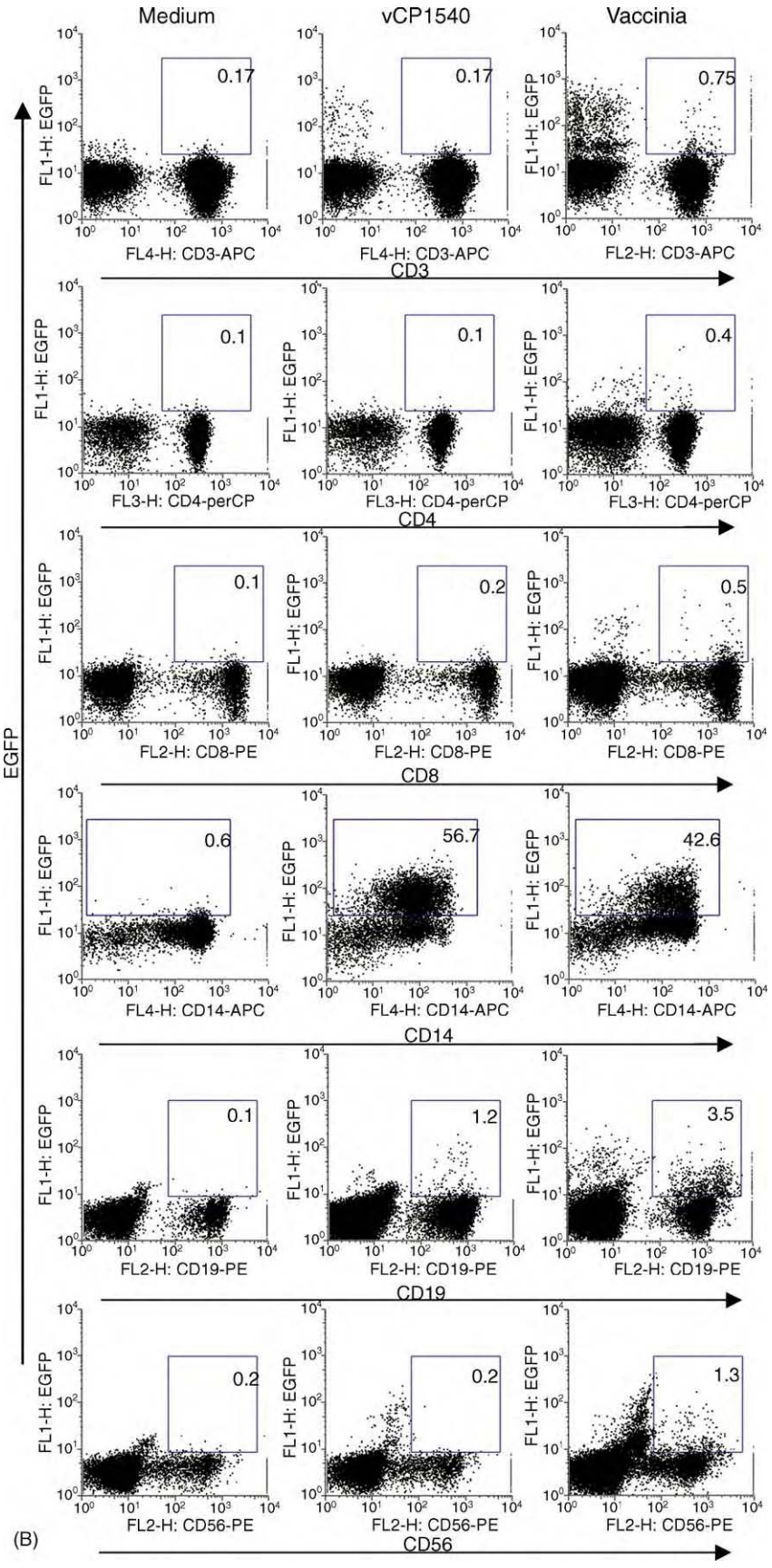

Fig. 1. (*Continued*)

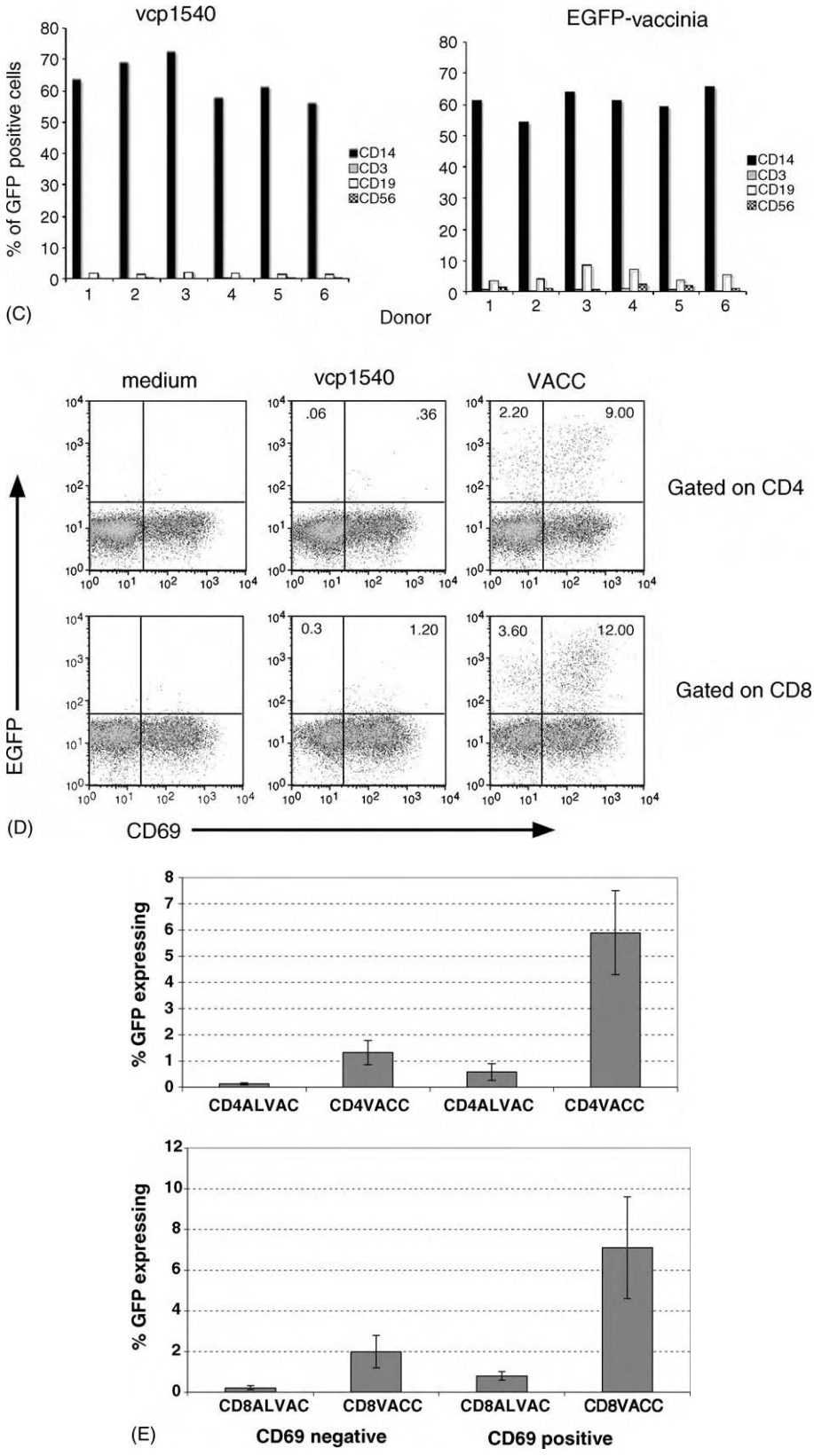

Fig. 1. (*Continued*).

Flow cytometric analysis with fluorochrome-conjugated mAbs specific for T cells, B cells, monocytes, or natural killer cells (NK cells) demonstrated that the recombinant EGFP-ALVAC virus (vCP1540) mirrored this marked preferential infection for peripheral monocytes. The kinetics of leukocyte infection with vCP1540 or EGFP-vaccinia virus typically observed are represented in Fig. 1A. EGFP-positive monocytes infected with vCP1540 comprised 1.6, 8.9, 56.7, 62.5, and 84.3% of total monocytes by 1, 2, 4, 8, and 24 h post-infection, respectively (means from six experiments). This closely paralleled the time-course of vaccinia virus infection, which showed 1.1, 5.6, 42.6, 59.3, and 89.3% EGFP-positive monocytes at 1, 2, 4, 8, and 24 h post-infection, respectively (means from six experiments).

Despite the similarity in kinetics of monocyte infection, notable differences in infectious cell tropism between vaccinia virus and ALVAC were observed. ALVAC demonstrated a lower ability to infect $CD19^+$ B cells (Fig. 1A–C). At 8 h post-infection with ALVAC, 1.2% of the $CD19^+$ B cell subpopulations were EGFP-positive compared to EGFP-vaccinia, which latter infected 8.6% of the B cell population. In addition, ALVAC was unable to infect *ex vivo* NK cells and T lymphocytes, including $CD3^+$, $CD4^+$, $CD8^+$ T cells, whereas EGFP-vaccinia was able to infect 2.6% of NK cells and a small percentage (under 1%) of T lymphocytes at 8 h post-infection (Fig. 1A–C, and E). Activation of $CD3^+$ T cells, either by stimulation with cross-linked anti-human CD3/CD28/CD49d mAbs, or Staphylococcal Enterotoxin B superantigen (SEB) exposure (data not shown), induced minimal changes in permissiveness to infection by ALVAC (Fig. 1D and E) compared to non-stimulated conditions, with minimal if any enhancement of infection in activated cells. However, more dramatic enhancement of infection by vaccinia virus was observed in activated $CD4^+$ and $CD8^+$ T cells (Fig. 1D and E), but not to the degree of infection as observed with monocytes. Activation was confirmed by showing strong expression of CD69 (Fig. 1D) or IFN-gamma (IFN-γ) on T cells after stimulation (data not shown). Similar to the *ex vivo* peripheral T cells or activated $CD3^+$ T cells, a human $CD4^+$ T cell line, CEM-T4, which constitutively expresses activation markers, such as Ox40 (data not shown), was non-permissive to ALVAC infection, but displayed very low level susceptibility to vaccinia virus infection (infection rate always <1%) (data not shown).

3.2. Preferential infection of ALVAC or vaccinia virus to monocytes correlates with the level of virus preferential binding

In order to distinguish whether viral tropism was dependent on cellular entry or due to lineage specific differences in the expression of cellular genes that are necessary for virus replication, studies in virus binding to cells of varying lineages were performed.

Robust binding of a virus to a cell surface is required for infection. Individual interactions between a viral contact site, and a single attachment factor, or receptor, can be weak, with binding constants as low as one [30]. However, when this is multiplied over numerous contacts, a virtually irreversible binding avidity can be realized. The observed preferential expression of vCP1540 and EGFP-vaccinia viruses in monocytes is the result of preferential binding to this population, rather than lineage-specific differences in the expression of viral genes (see Fig. 2). This was determined by exposing ALVAC or vaccinia virus at an MOI of 10 for 1 h to PBMCs, washing off free virus, and then immediately sorting cells into various subpopulations, including $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, and $CD56^+$ NK cells. Binding of virus to cell subpopulations was assessed by employing PCR and dot blot hybridization from sorted cells in order to detect the presence of ALVAC or vaccinia virus DNA. Overall, PCR data correlated with the infection data obtained by flow cytometric analysis. $CD14^+$ monocytes demonstrated high susceptibility to ALVAC or vaccinia virus binding. $CD19^+$ B cells exposed to ALVAC displayed only low level susceptibility. Compared with the binding to ALVAC virus, $CD19^+$ B cells exposed to vaccinia virus displayed a higher level of positive PCR signals. Both $CD3^+$ T cells and $CD56^+$ NK cells showed negative PCR for ALVAC virus binding, however, low level PCR signals for vaccinia virus binding were observed (Fig. 2A and B). Dot blot hybridization showed similar patterns as depicted with PCR, confirming strongest binding of ALVAC or vaccinia virus to $CD14^+$ monocytes. Similarly, $CD19^+$ B cells displayed low level binding for ALVAC, and higher level for vaccinia virus. $CD3^+$ T cells and $CD56^+$ NK cells were negative for ALVAC but demonstrated very low level vaccinia virus binding (Fig. 2A and B).

3.3. ALVAC and vaccinia viruses bind rapidly to monocytes and $CD14^{Hi}$ monocytes are early targets

The standard assay for the detection of *in vitro* infection of target cells with poxviruses, including ALVAC and vaccinia viruses, allows 1 h at 37 °C for adsorption of the virus to target cells. This is followed by removal of unbound virus particles by two washes with 2% FBS/PBS. The virus-exposed cell pellets are subsequently resuspended in cell culture medium, and incubated at 37 °C for various time intervals. Using this common method, ALVAC and vaccinia viruses infected $50.4 \pm 6.3\%$ ($n = 6$) and $40.2 \pm 4.9\%$ ($n = 6$), respectively, of human peripheral monocytes at the 4 h post-infection (Fig. 1A and B). It became apparent to us, however, that a significant proportion of ALVAC and vaccinia viruses bound to monocytes extremely rapidly. In a representative experiment, the adsorption window was reduced from 1 h to only 30 s. Free virus particles were then removed by extensive washing (five times in 3–4 ml of wash buffer), followed by incubating at 37 °C for 4 h with the adsorbed viruses. This abbreviated protocol allowed for infection of 44 and 17% of

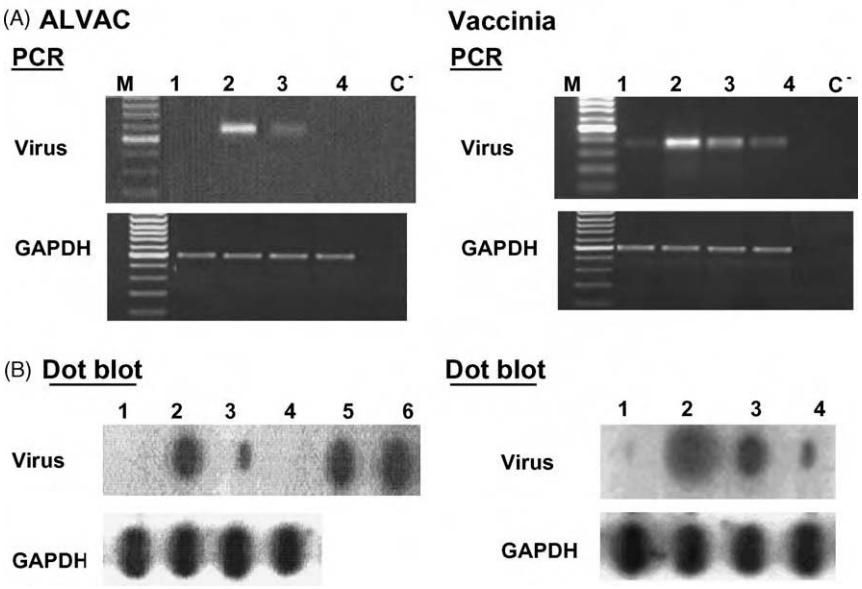

Fig. 2. Preferential infection of ALVAC or vaccinia virus to monocytes correlates with the level of virus binding. vCP1540 or EGFP-vaccinia virus was absorbed to PBMCs at an MOI of 10 for 1 h at 37 °C in an environment of 5% CO_2. After removing unbound virus particles by washing twice with ice-chilled 2% FBS/PBS, virus-exposed PBMCs were stained with fluorochrome-conjugated Abs of CD3, CD14, CD19, or CD56, and then subjected to fluorescence-activated cell sorting. Sorted $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, and $CD56^+$ NK cells were subjected to DNA isolation for PCR and dot blot hybridization. (A) Ethidium bromide-stained PCR-amplified ALVAC or vaccinia virus sequences from DNA samples prepared from sorted PBMC subsets. M: GeneRuler 100 bp DNA ladder plus (MBI Fermentas); 1, 2, 3, 4: sorted $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B lymphocytes, and $CD56^+$ NK cells, respectively; C^-: PCR negative control. The specificity of PCR for both ALVAC and vaccinia viruses was confirmed by DNA sequences of the PCR products. Shown is one representative experiment of three from three different donors. (B) Dot blot analysis of ALVAC or vaccinia virus DNA in sorted PBMC subsets. 1, 2, 3, 4: sorted $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B lymphocytes, and $CD56^+$ NK cells, respectively; 5, 6: 10 ng of DNA of purified PCR products or plasmid DNA of cloned PCR products generated from purified ALVAC virion served as positive controls. Oligonucleotide GAPDH.For (5′-CCTGGCCAAGGTCATCCATG-3′) was labeled with DIG-ddUTP and used as a probe for detecting human house keeping gene GAPDH as the control of the DNA quality and quantity. Shown is one representative experiment of three from three different donors.

monocytes by vCP1540 and EGFP-vaccinia viruses, respectively, with only 30 s virus adsorption time, whereas 57 and 42% EGFP-positive monocytes were infected by ALVAC and vaccinia, respectively, after 1 h adsorption (Fig. 3A). Thus, a 30 s adsorption period allowed for 77.2% of the ALVAC binding, and 40.5% of the vaccinia virus binding, observed over the full 1 h period.

Having observed a strong cellular infective tropism towards peripheral monocytes by both the ALVAC and vaccinia viruses, we sought to determine whether a particular subpopulation might be especially vulnerable. Peripheral monocytes were stained with $CD14^{APC}$ Ab at multiple time points post-infection with vCP1540 or EGFP-vaccinia virus. It was observed that both ALVAC and vaccinia viruses appeared to predominantly infect the $CD14^{hi}$ subset of monocytes at the early time points of 1 or 2 h post-infection. At these two time intervals, 1.5 and 4.6% of $CD14^{hi}$ monocytes were EGFP-positive from vCP1540 and 1.7 and 2.1% of $CD14^{hi}$ monocytes were EGFP-positive from EGFP-vaccinia. Infection among the $CD14^{dim}$ population remained very low (0.1% EGFP-positive from vCP1540 infection and 0.1–0.5% EGFP-positive from EGFP-vaccinia virus infection) at 1 and 2 h post-infection. This disparity between infection efficiency of high and low CD14 expressing cells had diminished by 8 h post-infection, at which time, both $CD14^{hi}$ and $CD14^{dim}$ subpopulations, were similarly infected by both viruses. $CD14^{hi}$ monocytes, however, still retained a significantly higher level of infection than their $CD14^{dim}$ counterparts, in with 41.6% *versus* 29.8% and 38.6% *versus* 29.1% of cells were EGFP-positive from vCP1540 and EGFP-vaccinia, respectively (Fig. 3B). Thus, monocyte infection by ALVAC and vaccinia viruses is highly dependent on CD14 expression. It is also possible that $CD14^{hi}$ cells become infected and then downregulate CD14, showing infection of $CD14^{dim}$ cells. We, however, did not observe any direct effect of vaccinia or vCP1540 infection on CD14 expression of monocytes, before and after infection (data not shown).

3.4. ALVAC and vaccinia viruses demonstrated different infection patterns to CD40L-matured human monocyte-derived dendritic cells

ALVAC virus has been shown to be capable of infecting both immature and mature human monocyte-derived dendritic cells (MDDCs) with a substantially higher frequency of infection to immature MDDCs. In previous work [22], a recombinant ALVAC virus vCP172 expressing SIV gag and pol was used and expression of both ALVAC and SIV antigens were monitored in the exposed MDDC populations. Consistent with this infection pattern, an EGFP-expressing

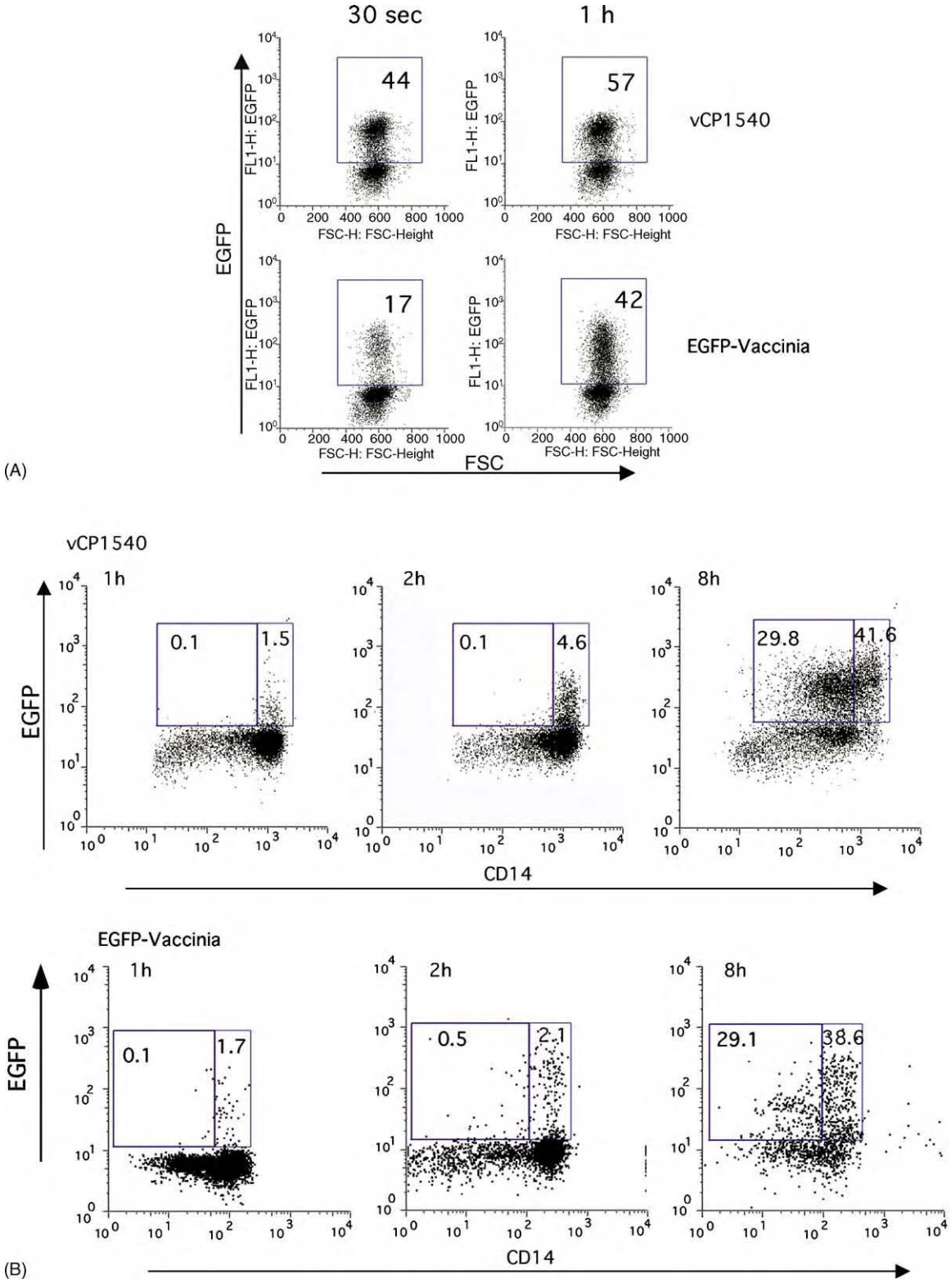

Fig. 3. ALVAC or vaccinia virus binding process is extremely rapid and $CD14^{Hi}$ monocytes are early targets. Purified monocytes were absorbed with vCP1540 or EGFP-vaccinia virus at an MOI of 10 at 37 °C for various time intervals (from 30 s to 1 h). Cells were then washed five times with a large amount of wash buffer (ice-chilled 2% FBS/PBS) for each wash. (A) Cell pellets from the final wash were suspended in the complete RPMI 1640 medium at $1–3 \times 10^6$ cells/ml and incubated in a 5% CO_2 incubator for 4 h. After incubation, cells were washed twice and directly subjected to flow cytometric analysis. The number in each dot plot shows the percentage of EGFP-positive cells out of the total cells. (B) Purified peripheral monocytes were infected with vCP1540 or EGFP-vaccinia virus for various time intervals (from 1 to 24 h), and then stained with $CD14^{PE}$ mAb and subjected to flow cytometric analysis. The number within each plot shows the percentage of the EGFP-positive monocytes in the cells expressing dim or high level of CD14 ($CD14^{dim}$ or $CD14^{Hi}$).

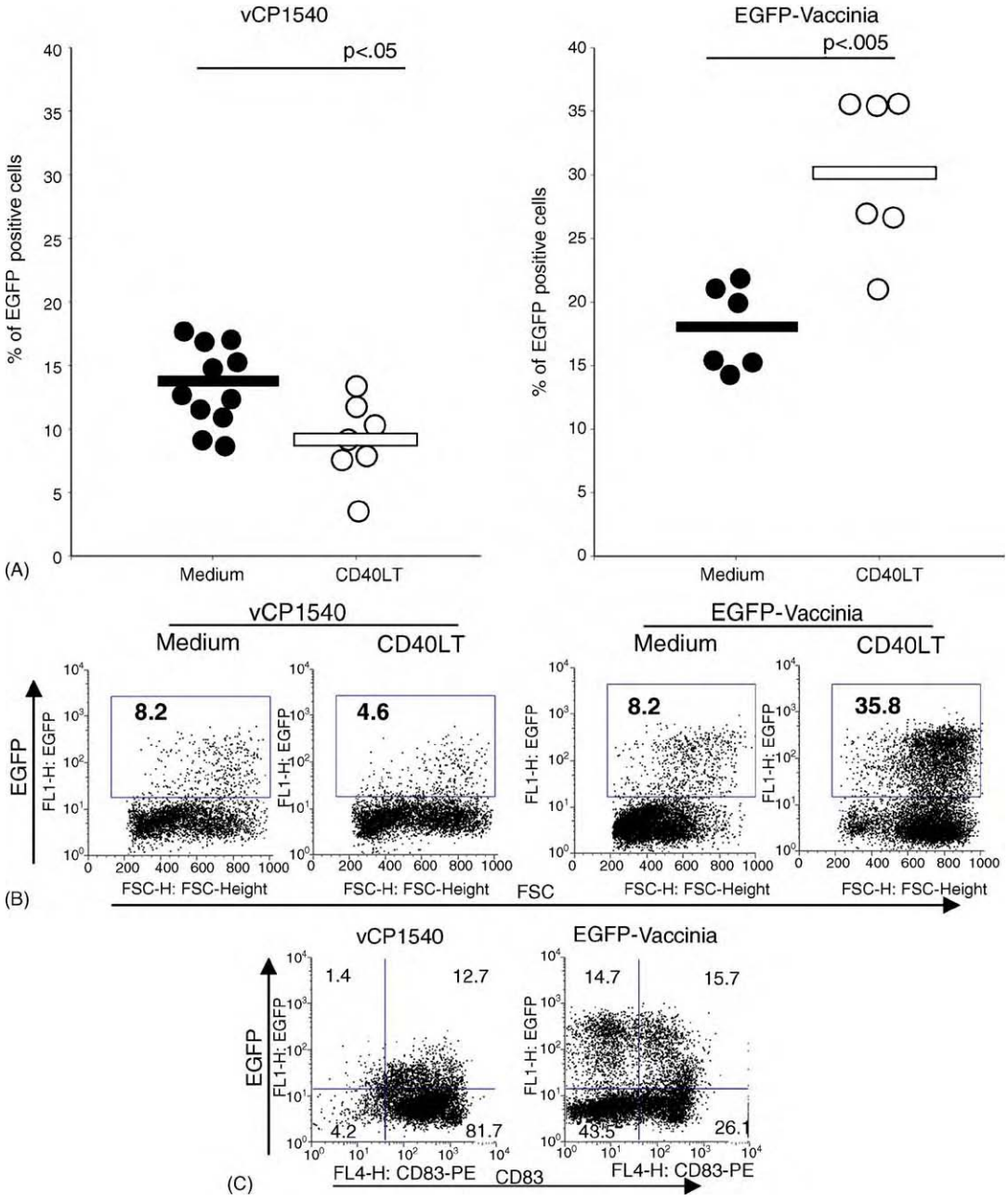

Fig. 4. ALVAC and vaccinia viruses demonstrate different infection patterns to CD40L-matured human monocyte-derived dendritic cells. Immature monocyte-derived dendritic cells (iMDDCs) were generated by culturing purified peripheral monocytes at 1×10^6 cells/ml in medium consisting of RPMI 1640 plus 10% FBS, 2 mM glutamine, 25 mM HEPES, and antibiotics in the presence of 50 ng/ml recombinant human GM-CSF and 100 ng/ml recombinant human IL-4. GM-CSF and IL-4 were added again on days 3 and 5 with the fresh complete RPMI 1640 medium. After 7 days of culture, the majority of cells were CD1a^{high}, MHC class II$^+$, CD80^{low}, and CD14$^-$, representing an immature DC (iDC) phenotype. The immature DCs were matured for 2–3 days by stimulation of soluble recombinant human CD40L trimer protein at a concentration of 2 μg/ml. Control immature DCs continued to be cultured in the presence of IL4/GM-CSF. For the infections, immature MDDCs and CD40LT-matured MDDCs were exposed to medium alone (no virus condition), vCP1540 or EGFP-vaccinia virus at an MOI of 10 for 1 h at 37 °C. Cells were washed twice with ice-chilled 2% FBS/PBS, and then cultured for 20 h at 37 °C in a 5% CO_2 incubator. Cells were then harvested and subjected to flow cytometric analysis. Part (A) demonstrates the infection patterns of immature (medium treated) and CD40L-matured MDDCs to vCP1540 and EGFP-vaccinia viruses. Part (B) is one representative experiment from at least six experiments, which shows the different infection patterns in immature MDDC (left plot) and matured MDDC (right plot). The number within each plot shows the percentage of the EGFP-positive MDDCs out of the total MDDCs. In (C) is a representative experiment showing expression of CD83 and infectivity of immature MDDC infected with vCP1540 (left plot) or EGFP-vaccinia (right plot). In (D) surface staining for analyzing regulation of DC maturation markers CD80, CD83, and CD86 in response to ALVAC or vaccinia virus infection. Isotype control, solid histogram; thin line, medium control; ALVAC, thick black line; vaccinia virus, thick grey line. Shown is one representative experiment of six from six different donors. In (E) intracellular staining for TNF-α 24 h after immature MDDC infected with ALVAC or EGFP-vaccinia virus, or medium control, and in (F), showing distribution of TNF-α in EGFP-positive and EGFP-negative DCs in the same experiments. Shown is one representative experiment of six from six different donors.

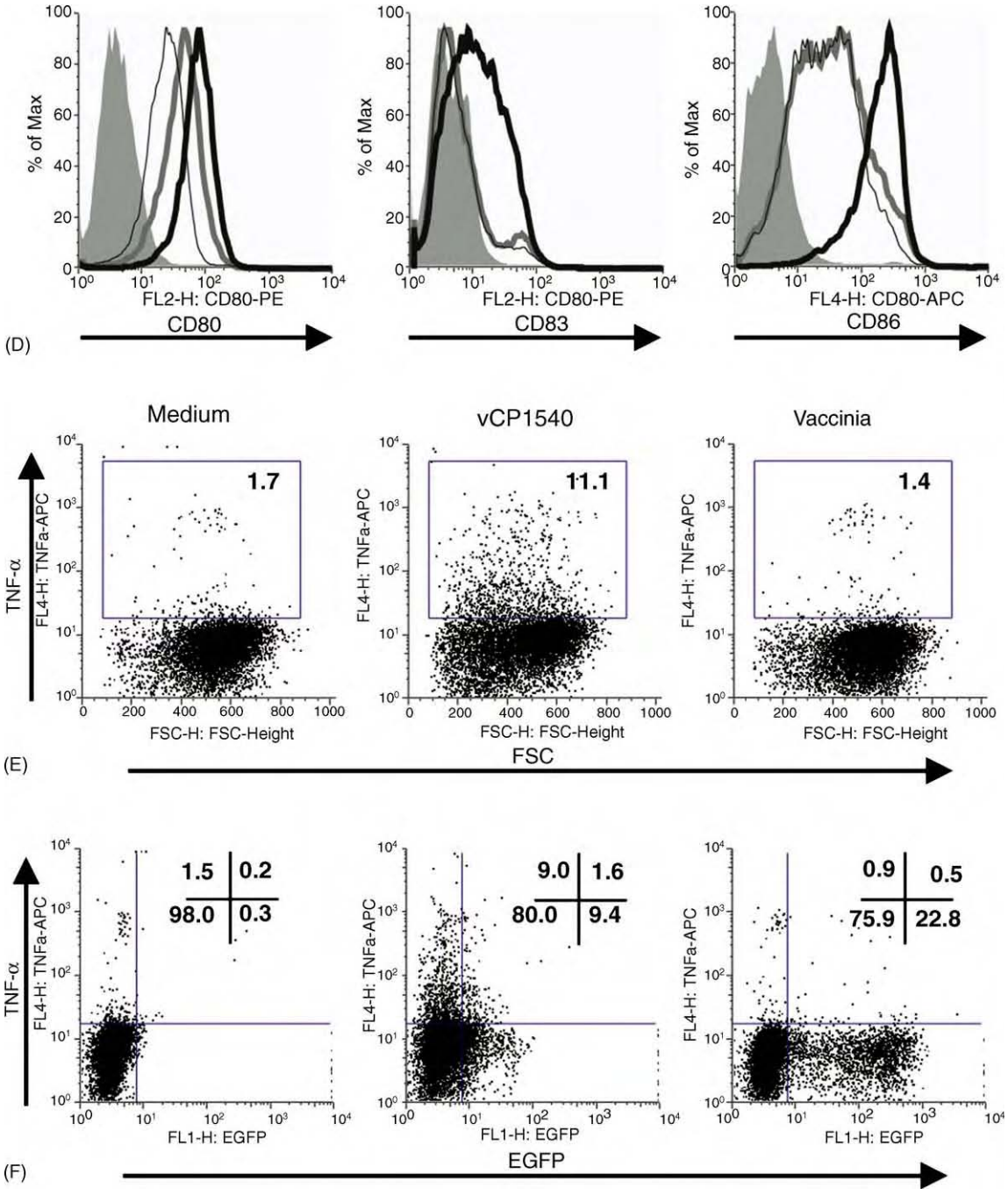

Fig. 4. (*Continued*).

recombinant ALVAC virus vCP1540 displayed infection of both immature and CD40LT-matured MDDCs with a greater rate of infection observed in the former population (Fig. 4A and B). Vaccinia virus showed an inversion of this preference. Both immature and mature MDDCs were infected to some extent, but CD40LT-matured MDDCs exhibited a higher frequency of vaccinia virus infection (Fig. 4A). In addition, monocytes were much more susceptible to infection by either ALVAC or vaccinia virus when compared to monocytes that were differentiated into immature or mature MDDCs. When directly comparing infection of MDDC to monocytes, ALVAC infectivity checked at 20 h post-infection, revealed the following hierarchy, monocytes > immature MDDC > mature MMDC (mean infection rate of 82, 13.9, and 6.1%, respectively, for three experiments) and for vaccinia virus; monocytes > mature MMDC > immature MMDC (mean infection rate of 82, 27.2, and 14.2%, respectively, for three experiments).

Previously, ALVAC infection could induce MDDC maturation measured as marked up-regulation of the DC

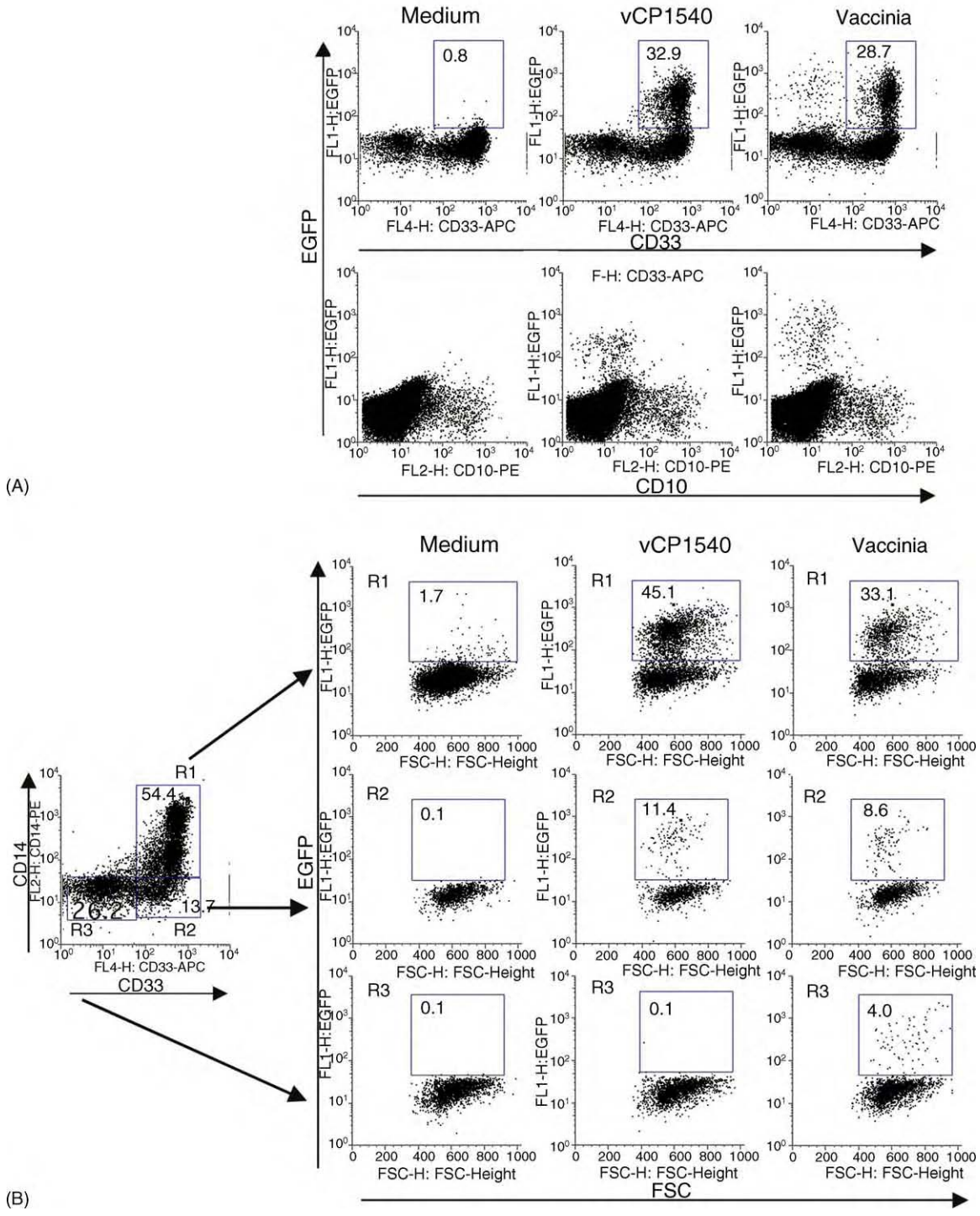

Fig. 5. ALVAC or vaccinia virus infects myeloid lineage cells but not lymphoid lineage cells in human bone marrow cell culture. Freshly isolated human bone marrow cells were infected with vCP1540 or EGFP-vaccinia virus at an MOI of 10 for 8 h at 37 °C. The susceptibility and degree of myeloid lineage cells and lymphoid lineage cells to ALVAC or vaccinia virus infection were monitored using flow cytometric analysis with fluorochrome-conjugated mAbs specific for myeloid cell-specific transmembrane glycoprotein $CD33^{APC}$ [31,50] and lymphoid cell-specific surface antigen $CD10^{PE}$ [51] (A). Infected bone marrow cells were also stained with $CD33^{APC}/CD14^{PE}$ in order to study the susceptibility of myeloid progenitor cells to ALVAC or vaccinia virus infection (B). The number within each plot shows the percentage of the EGFP-positive monocytes in the cells expressing specific cell markers. One representative experiment shows the infection patterns of bone marrow cells from tested three healthy bone marrow donors.

maturation markers CD80, CD83, and CD86 as well as CD25 and DC-LAMP [22]. Generation and up-regulation of secreted TNF-α by immature DCs in response to infection with ALVAC was previously shown also to contribute significantly to DC maturation [22]. With a side-by-side comparison of DC maturation and TNF-α production in response to ALVAC and vaccinia virus infection, we found that vaccinia virus infection could not induce DC maturation as was observed in ALVAC infection (Fig. 4C and D). Intracellular staining with a combination of anti-human TNF-α^{APC} and IL-12^{PE} mAbs showed that significant TNF-α production occurred within 24 h of exposure of immature human DCs to ALVAC virus, while IL-12 production was not increased (Fig. 4E and data not shown). Interestingly, up-regulation of TNF-α production occurred within both EGFP-positive and EGFP-negative DCs in response to ALVAC infection, suggesting that the uninfected DCs might be activated by secreted cytokines or by direct cell surface contact with infected DCs (Fig. 4E). In contrast to ALVAC infection, vaccinia virus infection of immature DCs could not induce TNF-α or IL-12 above control conditions (Fig. 4E and data not shown).

3.5. ALVAC and vaccinia viruses infect myeloid lineage cells but not lymphoid lineage cells from human bone marrow

Within the peripheral blood population, ALVAC showed an almost exclusive cellular infective tropism for $CD14^+$ monocytes. Vaccinia virus displayed a broader, but related tropism, with infection being confined to leukocytes. Combined with the ability of both of these viruses to infect immature and mature MDDCs implies that poxviruses, including canarypox virus (ALVAC) and vaccinia virus, may have a preferential tropism for cells of the myeloid lineage. In order to test this hypothesis, freshly isolated human bone marrow cells were infected with vCP1540 or EGFP-vaccinia. The degree of EGFP expression in cells of myeloid (CD33) [31] *versus* lymphoid lineage (CD10) was monitored by flow cytometric analysis. Canarypox virus infection was strictly contained within the $CD33^+$ cell population, while vaccinia virus showed a strong, but not exclusive, preference for these cells.

EGFP expression in vCP1540 infected cells was observed exclusively in the $CD33^+$ myeloid cell population at levels ranging from 26.8 to 34.8% 8 h post-infection in the three bone marrow samples tested. ALVAC infection was not detectable in $CD10^+$ cells (Fig. 5A). This myeloid lineage tropism preference was also mirrored in vaccinia infection, albeit with a lower infection rate of $CD33^+$ population (19.6–28.7% from three healthy bone marrow samples tested). In contrast to ALVAC, vaccinia was able to infect $CD33^-$ cells to a small extent (1.1–1.3% of $CD33^-$ cells were EGFP-positive at 8 h post-infection) (Fig. 5A). $CD33^+/CD14^{dim}$ contains myelomonoblasts, whereas, $CD33^+/CD14^{hi}$ cells represent cells committed to monocyte differentiation [32]. Interestingly, both ALVAC and vaccinia viruses exhibited a significantly higher level of infection to $CD33^+/CD14^{hi}$ than their $CD33^+/CD14^{dim}$ counterparts, with 45.1% *versus* 11.4% and 33.1% *versus* 8.6% of EGFP-positive cells from vCP1540 and EGFP-vaccinia virus infections, respectively (Fig. 5B). Both canarypox virus and vaccinia virus therefore exhibited an infective tropism for human monocytic lineage cell types, particularly after the myelomonoblast stage of differentiation. Canarypox viral infection was strictly contained within the $CD33^+$ cell population, while vaccinia virus showed a strong, but not exclusive, preference for these cells.

4. Discussion

The principal finding of the present work is that both canarypox and vaccinia viruses exhibited preferential tropism for human monocytic lineage cell types ranging from a common myelomonocytic precursor to terminally differentiated mature MDDCs. Canarypox viral infection was strictly contained within the myelomonocytic cell-specific $CD33^+$ cell population in bone marrow cells, while vaccinia virus showed a strong, but not exclusive, preference for these cells. More specifically, tropism of both viruses was strongly biased toward cells with monocytic differentiation, *i.e.*, CD14 expression. ALVAC demonstrated a diminutive ability to infect $CD19^+$ B cells but was unable to infect other cell types whereas vaccinia virus infection was observed in the B lymphocytes and NK cell populations to certain levels, minimally in resting T cells, and generally to a much lesser extent than that of monocytes. In keeping with the recent study by Chahroudi et al. [23], we also observed some enhancement of infection ability of vaccinia virus to T cells that were activated through CD3/CD28/49d mAbs crosslinking, consistent with the upregulation of a receptor on activated T cells that allows virus entry. These findings suggest that both viruses use a common receptor on monocytoid cells, but that vaccinia virus may use other receptors to expand its tropism.

The observed preferential infection of ALVAC or vaccinia virus to monocytes is the consequence of viral preferential binding to this population, rather than lineage-specific differences in the expression of viral genes, such as interferon, which would down regulate viral expression. This was determined by employing PCR and dot blot hybridization to detect viral DNA, not RNA, from DNA preparations of $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, and $CD56^+$ NK cells sorted from ALVAC- or vaccinia virus-exposed PBMCs. In addition, ALVAC and vaccinia both preferred to bind to $CD14^{hi}$ *versus* $CD14^{dim}$ monocytes resulting in a more rapid infection of the former cell population. $CD14^{dim}$ monocytes are a subpopulation of monocytes that have differentiated in response to various inflammatory stimuli [33]. Although the exact function of $CD14^{dim}$ monocytes remains to be established, they have been shown to be expanded in peripheral

blood in sepsis, arthritis, and HIV infection [33]. Our data show that both populations are susceptible to ALVAC and vaccinia, indicating that both quiescent and activated monocytes can be targets of these viruses. The cellular receptors which ALVAC is binding on monocytes appears to be correlated with the level of CD14 expression. Candidate receptors for ALVAC on monocytes is an area of investigation which we are currently pursuing.

It is possible that the differing tropisms of ALVAC and vaccinia viruses may explain in part the reduced reactogenicity of ALVAC when used as an immunogen. Canarypox preferentially infected monocytes which tend to have a shorter life span (days to months) and barely infected long-lived B lymphocytes and did not infect T lymphocytes and NK cells. Although we did not directly test for productive infection of ALVAC in monocytes, previous studies have shown that ALVAC undergoes a non-productive abortive infection in human cell lines [12,34]. In addition, it has also been reported that vaccinia virus infection of monocytes/macrophages and dendritic cells is abortive [35–37]. In contrast, vaccinia virus infected a larger spectrum of cell types, and in particular has been recently shown to maintain a productive infection in activated human T cells [23]. As T and B cells can be long-lived (years), it is possible that this combined feature of greater tropism and productive infection in a subpopulation of T cells and B cells may propagate infection away from local sites of inoculation. Although, further dissemination of vaccinia may enhance antigen presentation, another concern to vaccinia vaccination would be greater reactogenicity due to enhanced inflammation in response to virus infection. In this regard, when ALVAC was evaluated as a vaccine vector, local reactions were common, but fewer than 2% of vaccines had severe local responses, and less than 1% experienced severe local pain or tenderness [38]. In contrast, vaccinia virus induces high fevers in >20% of vaccinated children, and is associated with rare complications including generalized vaccinia infection, and post-vaccinial encephalitis [39]. In addition, the potential for vaccinia to disseminate in memory T cells and B cells which are long-lived may have relevance to the ability for vaccinia virus infection to persist in the host. Given this, vaccinia virus has been avoided in immunocompromised hosts, whereas, ALVAC has been given to immunocompromised individuals without sequelae [40,41]. The role of vaccinia infection on the function of the T cells and B cells, and the role of abortive infection of vaccinia and ALVAC on monocyte function *in vivo*, is an area which requires further study.

Both canarypox and vaccinia viruses were able to infect mature and immature MDDCs. Interestingly, they displayed an inverse infection preference for the two subpopulations. ALVAC showed a higher affinity for immature cells, which is consistent with a previous report [22], while vaccinia virus infection rates were higher in their matured counterparts. ALVAC infection could induce MDDC maturation whereas vaccinia virus infection did not. Generation and up-regulation of secreted TNF-α by immature DCs in response to infection with ALVAC contributed significantly to the DC maturation [22]. We could reproduce this result using an EGFP-expressing recombinant ALVAC virus vCP1540. Interestingly, up-regulation of TNF-α production occurred within both EGFP-positive and EGFP-negative DCs in response to ALVAC infection, suggesting that the uninfected DCs might be activated by secreted cytokines or by direct contact with infected DCs. Further studies exploring the mechanism of TNF-α induction in uninfected DCs, including the role of Toll-like receptors are clearly warranted. It was noted that CD83, a maturation marker, was down-regulated in response to vaccinia virus infection, which is in agreement with previous studies showing that vaccinia virus inhibits the maturation of human DCs [35]. Vaccinia virus infection did not stimulate DCs to generate either TNF-α or IL-12, two key cytokines in the induction of DC maturation [42–44]. Furthermore, production of cytokine receptor homologues in the early cycle by vaccinia virus, *e.g.*, for IFN-α, may account in part for the block in the DC maturation [35], as IFN-α can also induce maturation of DC. The practical relevance for the interactions of these viruses with DCs is currently unclear. In the context of traditional vaccination, ALVAC may be more advantageous than vaccinia. Since immature DCs are found in the peripheral tissues where a vaccine would be introduced, ALVAC would have a greater ability to mature these DCs as they migrated to draining lymph nodes, whereas vaccinia would have a tendency to inhibit their maturation.

Furthermore, with the hope of engineering better vaccines, new strategies are being used in an attempt to directly target DCs by live viral vectors in order to load antigens on DCs. Recent advances in techniques for the cytokine-induced differentiation of monocytes into DCs have provided easier access to these important antigen presenting cells, which had been a scarce cell type since their discovery 30 years ago [26]. Many clinical studies using autologous DCs loaded with tumor antigens are in progress [45–47]. These DCs induce antitumor immunity such as expanded cytotoxic T cell (CTL) activity [45,46]. In healthy human volunteers, a single injection of mature MDDCs loaded with multiple antigens (*i.e.*, tetanus toxoid, influenza matrix peptide, and keyhole limpet hemocyanin) expands both $CD4^+$- and $CD8^+$-specific responses [48]. Studies show that the level of DC maturation may be of critical importance in the nature of the immune response to DC-delivered antigens [49]. Recent studies suggest that antigen-loaded mature DCs induce a protective immune response, whereas antigen-loaded immature DCs may "silence" the immune response *via* expansion of IL-10 producing T cells [13]. Compared with vaccinia virus, ALVAC is able to infect both immature and mature DCs and also markedly induces maturation of both infected and uninfected immature DCs. Thus, ALVAC has more potential to be used to load antigens of tumor or infectious agents to immature and mature DCs which is of special interest in the field of vaccine development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2006.06.011.

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APPENDIX 4.

HIV infection and dementia in older adults

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HIV Infection and Dementia in Older Adults

Victor Valcour and Robert Paul

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Abstract
Human immunodeficiency virus (HIV) infection in older patients is becoming increasingly common as seropositive individuals live longer because of long-term antiretroviral treatment. Simultaneously, the development and expression of dementia among HIV-infected patients is evolving in the era of highly active antiretroviral therapy (HAART) and immune reconstitution. How long-term HAART interacts with chronic HIV infection and advanced age with regard to cognition is not fully understood. This article provides an overview of HIV cognitive impairment as it relates to aging and presents some emerging issues in the field. Particular emphasis is placed on describing the changing landscape of HIV-related cognitive impairment and discussing possible concerns regarding the long-term effects of antiretroviral treatment. A brief discussion of potential adjunctive therapies to reduce cognitive symptoms associated with HIV infection in older individuals is provided.

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APPENDIX 5.

The Dengue Fever outbreak in Hawaii: A bioterrorism model for vector-borne illnesses

The dengue fever outbreak in Hawaii: a bioterrorism model for vector-borne illnesses

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Abstract: Dengue fever is a flu-like illness of viral origin transmitted via various day-feeding mosquitoes. Annually there are approximately 20 million cases worldwide with 24,000 deaths. Hawaii's Dengue Fever outbreak in 2001 came during a terrorism national attack providing insight into a possible vector-borne bioterrorism (BT) event. The dengue model is valid, as the National Institutes of Health has upgraded dengue fever as a potential BT threat. The Hawaii experience makes it possible to list strengths/capabilities necessary for dealing with such biological events and relate this to the National state of BT preparedness.

Keywords: bioterrorism; dengue fever; disease outbreak; Hawaii; technology; telemedicine.

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1 Introduction

The story of Hawaii's 2001 dengue fever outbreak is an important one – the situations, deficiencies and solutions experienced by Hawaii during this time can be applied to our thinking regarding the nation-wide push for effective bioterrorism preparedness (bioterrorism can be defined as the intentional use of microorganisms or toxins derived from living organisms to produce death or disease in humans, animals, or plants (Lathroop and Mann, 2001)). In many ways the 2001 episode in Hawaii can serve as an interesting bioterrorism model for a complex 3-way terrorism event with the major spoke being of biological origin. Even though the main terrorist in this case was Mother Nature, the atmosphere of terrorism was strongly present due to the Dengue Fever outbreak occurring synchronously with the 11 September tragedy.

It is important to note that prior to the 11 September crisis, terrorism activities were of a different nature. Generally, terrorists planned and executed their events to make a vivid impact and gain attention with forceful or violent means, to protest policies or communicate their beliefs. Post 9/11 the terrorist goals are now to kill as many people as possible and also bring down a nation economically (we can call this 'modern' terrorism) (Holton, 2002). They will obtain the necessary education to do it (such as learning to fly a commercial aircraft and possibly even to obtain college degrees and expertise in microbiology and techniques necessary for a thoughtful and sophisticated biological attack on a nation). We know now that they will take the necessary time to obtain this knowledge and eventually execute their goals as planned. We also learned that multiple attacks performed simultaneously are a distinct possibility and a successful strategy that can dilute the emergency relief response, manpower and supplies, which in turn can result in a greater number of casualties, prolonged suffering and panic and a prolonged recovery.

A major lesson from 11 September was that the unthinkable can happen – terrorists educating themselves (through pilot school) to achieve seven simultaneous disasters (four crashed airplanes, two collapsed buildings in New York, and substantial damage to the Pentagon). The question regarding bioterrorism now is: are terrorists learning biological sciences in capable schools, possibly with access to infectious disease material that they can, with proper technique, replicate and spread using various dissemination modes? Much of the literature on bioterrorism caution that it is not a matter of 'if' it will happen but 'when' it will happen. We currently have a window of time to prepare and Nature, with her periodic infectious disease outbreaks, has provided important experiences for us to find out our deficiencies in dealing with major biological events and find solutions.

The dengue fever outbreak in Hawaii was a valuable and unique experience in that it was actually a multiple crisis – perhaps similar to what might be encountered in the future given the 'modern' terrorist thinking discussed above (affecting masses of people, affecting the economy, several crises all happening simultaneously). Although the dengue outbreak actually started in May of 2001 (the Hawaii State Department of Health (DoH) found this out retrospectively) the realisation of an outbreak occurring happened on

12 September 2001. During this time the nation was in crisis from the multiple World Trade Center/Pentagon attack. Also at this time the anthrax-laced letters were appearing and infecting people (a crisis that Hawaii post offices had to deal with also) (Global Security Newswire, 2001). Thus, there existed a three-pronged emergency, complete with the grounding of aircraft resulting in a complicated and isolated situation for Hawaii.

The 2001 dengue fever outbreak in Hawaii is a good example of how outbreaks can occur (in this case brought by non-terroristic foreign travelers) and progress in a subtle manner before being detected and dealt with. The dengue model is valid for bioterrorism preparedness, as the National Institutes of Health has upgraded dengue fever as a potential bioterrorism threat. This paper will discuss the nature of dengue fever, its history and emergence in Hawaii, and the 2001 Hawaii dengue fever outbreak experience – including the unexpected difficulties and findings, disadvantages/deficiencies, short-term and long-term solutions. We will then list strengths and capabilities necessary for dealing with a vector-borne biological event and relate the overall Hawaii experience to the national state of preparedness.

2 The nature of dengue fever

Dengue fever (DF) is a flu-like illness characterised by symptoms such as high fever, severe headache, pain behind the eyes, muscle and joint pains and rash. The virus is transmitted via the bite of various day-feeding mosquitoes. With DF there is a mosquito-human transmission with humans serving as reservoirs for the disease. There is no human-human spread of disease. Here the 'enemy' is the mosquito and defences against the mosquito are key in protecting human health. Eradication of the mosquito is key to stopping the outbreak. There is currently no vaccine. DF has increased in both incidence and distribution over the past 40 years. Annually it is estimated that there are 20 million cases of dengue infection worldwide, resulting in 24,000 deaths (Dengue fever outbreak GIS Program).

In 1944 Albert Sabin successfully isolated the virus that causes DF and found that it belongs to the Flavivirdae virus family. There are more than 70 known members of the Flavividae family. Some examples include Yellow Fever and Japanese Encephalitis Virus (Gubler, 1994). Flavivirdae are viruses that utilise humans, lower primates and mosquitoes as hosts. The dengue virus mainly relies on the mosquito Aedes aegypti as a vector to transmit it to human and primate hosts. Because dengue is an arthropod-borne virus it is also classified as an arbovirus. The *A. aegypti* mosquito is an urban mosquito that thrives in pools of standing water. Peak transmission is associated with increased amounts of rainfall and mosquito density. Therefore tropical climates are ideal for the mosquito to survive. Pools, puddles, buckets of water, gutters and people spending significant amounts of time outdoors aid in successful transmission of the virus.

Presently there are four known serotypes of dengue virus. These are labelled:

- DEN-1
- DEN-2
- DEN-3
- DEN-4.

The different serotypes have the same morphology and genome; however, each serotype displays different antigens. Historically, DEN-2 is the prevalent serotype found in Southeast Asia and may be responsible for immunity against Yellow Fever. DEN-3 has been found in the Caribbean and DEN-1 has been found in the Pacific Islands (Hawaii, Marshall Islands). Exposure to any one of the dengue virus strains confers immunity to reinfection by the same strain. However, if a person once infected becomes re-infected with a different strain, there is an increased likelihood that the virus will be expressed as a much more severe form of the disease, dengue hemorrhagic fever (DHS), which has been compared to ebola in its symptoms, or dengue shock syndrome (DSS), characterised by extremely low blood pressure. Both conditions can be fatal and require immediate medical attention (Gubler, 1994, 1997).

3 History of dengue fever

The first recorded cases of DF occurred in 1779 in Batavia, Indonesia, and Cairo, Egypt. In 1780 a dengue epidemic was reported in Philadelphia, Pennsylvania. For the past 200 years pandemics have been recorded in tropical and subtropical climates at ten to 30 year intervals. The intervals of several decades between outbreaks occurred because the virus was not as rapidly transported as it is today. After WWII, with increased travel and consequent spread of dengue viruses throughout the Pacific region and the Americas, the distribution of DF has changed. The first epidemic of DHF occurred in 1953 in Manila and the disease remained endemic in Southeast Asia for 20 years until it spread westward into the Indian subcontinent and China in the 1980s and 1990s. DHF and DSS are now leading causes of hospital admissions and death in Asian children (Dubler, 1994, 1997).

3.1 Dengue in Hawaii

The first recorded case of dengue in Hawaii occurred in 1893, when it was known locally as ‘boohoo fever’, a name arising from the emotional distress that usually accompanies the disease. The first widespread epidemic of dengue appeared in 1903, when approximately 30,000 people were infected. Another epidemic lasted from 1912 to 1915. Not until 1943 did the virus reappear in an outbreak that infected about 1500 people – and killed three – before it ran its course in 1945. The outbreak even prompted the closure of Waikiki. Experts suggest that the infection may have been transmitted from Fiji, which was experiencing an epidemic at the time. It was believed the virus was brought to Hawaii through US servicemen who had travelled through the South Pacific. The epidemic in the 1940s led to an effort, supported by federal funds, to eradicate the vector, Aedes aegypti. This mosquito was eliminated from most areas, although it still remains in parts of Moloka’i and the coast of West Hawaii from Kawaihae to Captain Cook. However, where Aedes aegypti was eradicated, Aedes albopictus quickly took its place (Environment Hawaii, 2001).

Since the last major outbreak, some cases of dengue have been reported in the state – including 18 from 1992–2000 – but all were among travellers who contracted the disease elsewhere. With an incubation period of from three to 14 days after initial exposure to the virus through a mosquito bite, an infected person can easily transport the virus unaware that he or she is a carrier. From about one day before the first symptoms

occur (fever, severe headache behind the eyes and debilitating joint pain) and for the next week, the infected person can pass the virus to a mosquito host. The mosquito passes the virus on to anyone it bites for the rest of its life, usually no more than three weeks (Environment Hawaii, 2001; Kitsutani, 2003).

4 The Hawaii 2001 dengue outbreak

Hawaii is considered the last domino in a DF epidemic that swept across the Pacific in 2001. Thousands of people were already affected by the virus in Palau, Samoa, French Polynesia, New Caledonia, the Cook Islands, and the Philippines months before (Pacific Islands Reports, 2001a–f). The virus appears to have arrived independently on Hawaii's various islands from Tahiti and American Samoa. It was the first indigenous outbreak of dengue fever in Hawaii since World War II (Bricking, 2001). Experts believe that a major reason for the global emergence of dengue fever is increased air travel between population centres in the tropics, allowing for the exchange of the virus and other pathogens.

Hawaii had a Type 1 dengue outbreak. Although most confirmed cases were in the Hana, Maui area, the virus had 'seeded' across the island, with a small number of infections in many places. Other cases were found on Oahu, and also Kauai to a lesser extent. Investigation of these sentinel cases on each island demonstrated that they were probably due to other travellers returning from the South Pacific, as opposed to people transporting the disease from Maui. Only 10% of the cases reported themselves to physicians. This population had a tendency to self-treat, lived alternative lifestyles and lived in very remote and isolated sites. For this reason dengue was in the area much earlier than September before the first case presented to a physician (retrospective analysis). Once the Department of Health team was onsite to deal with the outbreak, they went door to door (dengue was spreading from house to house). Experienced health personnel who have seen dengue before in other countries (Brazil) and worked to contain outbreaks elsewhere were involved in Hawaii's outbreak. This was very fortuitous since these experts recognised dengue early and convinced the population that mosquito eradication measures must be taken. During their investigations, they observed that the vector in this outbreak was not the usual *Aedes aegypti*, but a less efficient one, *Aedes albopictis*. This was an unexpected finding which puzzled investigators initially (Hurley, 2001a). The mosquito concentration in Hana at that time was thought to be particularly dense by health department field agents.

When the first suspected case of dengue reported to the Maui district Health Office (September 12, 2001), it was during the nationwide anthrax scare. Like other states, Hawaii had to respond to reports of suspected anthrax discoveries in the weeks following the September 11 attacks. Also during this time, the Centers for Disease Control and Prevention (CDC) was preoccupied with the anthrax scare and was not able to ship laboratory supplies to Hawaii so dengue cases and infected mosquitoes could be quickly diagnosed. Instead, blood samples and mosquitoes had to be shipped to a CDC laboratory in San Juan, Puerto Rico, resulting in a turn-around time of weeks (Starbulletin, 2002). The situation was unexpected. Hawaii received inadequate assistance for the dengue outbreak because too much was happening simultaneously elsewhere. Therefore, the lesson learned here is that if a biological event occurs in multiple regions of the US, the local and State levels should be prepared to respond.

During the Dengue outbreak, the Hawaii DoH had trouble storing samples to be sent to the CDC Dengue Fever laboratory in Puerto Rico. Due to the airplane grounding delay from 11 September, Hawaii did not have a true dengue diagnosis until three weeks later. At this point it was clear that Hawaii DoH should have its own analysis facility. In the meantime, as a quick alternative, some field diagnostic test kits were ordered (which also had to be shipped). By 19 September the diagnostic kits arrived and all of the initial blood samples tested positive. On September 21, CDC confirmed the field tests. During the three weeks of waiting for laboratory results spraying of insecticide around homes was done. Spraying was initiated within one week of when the first patient arrived to a physician. Therefore the Hawaii DoH was able to implement its major strategy within the week to deal with eradicating Dengue, which included public education (through TV and community meetings) and mosquito habitat reduction (ADHS; Altonn, 2001). Table 1 provides a chronology of events spanning one month after the first patient was examined.

Table 1 Chronology of initial events of the 2001 Hawaii dengue outbreak (Altonn, 2001; Kitsutani, 2003)

Sept. 8	A state surveillance program began a few days before the first suspected DF case
Sept. 12	First suspected case of DF reported to Maui District Health Office (DHO). 47 y/o female from Lower Nahiku (Hana), with fever, headache, myalgia, arthralgia, rash on palms/soles/legs, no recent travel history, WBC = 1,300; platelet = 50,000; husband and son with similar illness, other community members with 'Tahitian Fever'
Sept. 13	Maui DHO investigates family of case in lower Nahiku
Sept. 14	Febrile illness alert issue statewide by DoH
Sept. 17	DoH investigators from Oahu arrive on Maui to assist
Sept. 19	Maui Health Lab reports anti-dengue IgM+ results
Sept. 20	Vector Control begins mosquito fogging in Nahiku. Sites were sprayed on Maui, Oahu, Kauai, and the Big Island
Sept. 21	CDC lab confirms Dengue infection in initial cases
Sept. 23	Health Advisory to Maui health care providers
Sept. 24	Active surveillance initiated on Maui (14 sites)
Sept. 28	Health Advisory to ER and hospital staff statewide
Sept. 30	CDC officials arrive to assist in investigation
Oct. 1	CDC isolates DEN-1 virus from sera of initial cases
Oct. 2	Active surveillance expanded to 28 sites statewide
Oct. 8	First non-Hana and Kauai cases confirmed
Oct. 9	Active surveillance expanded to 51 sites statewide
Oct. 12	First Oahu case confirmed

4.1 Communications/media

During the outbreak, communication action taken by the Hawaii Department of Health (Hawaii Department of Health; Hawaii DOH, 2001) and the Maui County Civil Defense Agency included information/brochures, press releases, travel advisories, advisories to

Health Care Providers and contact numbers on their websites. Local newspapers provided information as to the progression/eradication of the disease in Hawaii (Anwar, 2003; Honolulu Advertiser, 2003; Hurley, 2001b; Kubota and Altonn, 2001; Wright, 2001a), progression of DF in other Pacific nations (Pacific Islands Reports, 2001a–f) and perspectives from health officials (Anwar, 2001a, 2003; Hurley, 2001c; Pacific Islands Report, 2001g; Wright, 2001b). Town meetings and a dengue phone line (initially receiving 1000 calls a day) were a key component to educate and inform the public (Pang, 2003).

The state and counties launched a massive public education, outreach and cleanup program to emphasise the importance of eliminating mosquito-breeding areas to prevent the spread of the virus. Television and radio public service announcements were launched to encourage residents to clean from their neighbourhoods any debris or containers holding water. State officials worked with the visitor industry to inform all arrivals of the outbreak. About 1000 tourists per day were receiving informational brochures and mosquito repellent from a tourist information site set up on the road to Hana. Three other roads into the area were closed because of high risk (Altonn, 2001). The Department of Health decided to inform the public on the threat and response taken knowing that the immediate effects on tourism might be negative but the long-term consequences would improve (Pang, 2003).

4.2 Technology utilisation

The use of technology was important in characterising the outbreak, identifying hot spots and communicating data and instructions. Health officials from the Centers for Disease Control and Prevention, the Hawaii State Department of Health and the Maui County Health Department teamed with the Pacific Disaster Center (PDC) to perform a joint analysis of the outbreak. The PDC was asked to use its Geographical Information System (GIS) and Global Positioning System (GPS) technology and capabilities to perform data collection, mapping and analysis in support of a wide range of activities throughout the planning, operational and analytical phases of the assessment process. Techniques used were Dengue Diffusion Patterns and Disease Vector Modelling (spatial model schema). PDC created maps for investigators in the field utilising remote sensing (vector control mapping) (Dengue fever outbreak GIS program, 2003; Chiesa and Napier, 2003). An important lesson learned was that GPS mapping was useful when used on a small town/village level to delineate ‘hot spots’ but became less useful on an island-wide level where normal maps would suffice (Pang, 2003).

A much needed intense exchange of information between a more Dengue-experienced country (Thailand) and a less-experienced location (Hawaii) took place on 17 October 2001 during a three hour telemedicine seminar entitled ‘Dengue Outbreak in Hawaii’. The seminar was an interactive videoteleconference between Honolulu, Hawaii and Bangkok, Thailand with presentations by Dengue experts from both locations. Attendees had the opportunity to dialogue with speakers at both sites. The video teleconference configuration was a high-speed link (over three ISDN lines) with excellent video and audio transmission and was part of a larger project known as THAI-HI (Thailand-Hawaii Assessment of Interactive Healthcare Initiative) funded by the Pacific Telehealth and Telemedicine Hui (Vincent et al., 2002).

Health officials working onsite in Maui later expressed that frequent use of telemedicine would have helped in consulting with other experts (information exchange would enhance better decision-making). Contacting experts in real-time and receiving news in real-time via telemedicine technology would have helped. The telephone was very useful and was used for verbal communications to find out regions where the DF was and regions where it was not. This helped to rapidly map the DF affected areas (Pang, 2003).

4.3 Factors that helped/hindered progress

There were several factors that delayed detection and action against the Dengue outbreak. These include (Anwar, 2001b):

- The remoteness of the setting (mainly concentrated in the Hana Maui area) that made communications difficult, with sporadic cell-phone coverage.
- The tendency for the affected population to self-treat illness. These people believed they had the 'Tahitian flu' and chose to treat it with their own herbal remedies. Only 10% of cases reported to a healthcare facility.
- Confirmatory diagnosis was delayed due to planes being grounded post 9/11, with no rapid diagnostic tests available in Hawaii.
- The community was reluctant to respond (to seek medical attention, allow the use of pesticides near their homes) until confirmatory tests had been done.
- Uncertainty regarding the progression the disease would take since the vector of this DF outbreak was not the usual one (*Aedes albopictus*) and considered an inefficient vector for DF.
- Although the military presence in Hawaii is an advantageous resource during a crisis or disaster, many military resources were not able to assist during the outbreak since all were standing by for homeland defense initiatives or deployment to Afghanistan.

Several advantages helped to balance the delays and prevented the outbreak from becoming worse:

- *Aedes albopictis* is an inefficient vector for DF. The situation was different during the World War II outbreak when *Aedes aegypti* infected thousands of people in Hawaii.
- Experienced personnel who have worked extensively with DF before were on staff at the Health Department. Their quick recognition of symptoms provided an early awareness.
- Technological help (modelling, mapping) was local and readily available (Pacific Disaster Center).

4.4 After the outbreak

The last reported case of DF in Hawaii was the week of February 3, 2002. In total, there were 119 confirmed dengue cases in the state from 27 May 2001 to 3 February 2002. The peak number of cases occurred in September–October 2001 (Hawaii DOH, 2002a). Although the outbreak has been under control for quite some time, the State Department of Health cautions that Hawaii will always be at risk for DF and must continue to control mosquito populations. The disease normally recurs during the warm summer months and anyone infected with the virus can easily bring it back to Hawaii from endemic areas in the world. It also can be easily established in Hawaii if residents fail to take precautions, including the continuing effort to control mosquito populations around their homes (Tanji, 2002).

The DF outbreak experience has resulted in a number of improvements in the health system. For example, on May 10 2002, the Hawaii State Department of Health unveiled plans for its long-term dengue fever management strategy. The plan included a long-term dengue surveillance system statewide, a statewide mosquito population survey, and ongoing vector control efforts (Hawaii DOH, 2002b). The statewide surveillance system includes ongoing sampling of patients who demonstrate dengue like symptoms. This provides the Health Department with valuable data, giving officials an opportunity to catch an outbreak before it spreads. In addition to monitoring the situation in Hawaii, the department will be keeping up to date with outbreaks around the world.

The statewide mosquito population survey was initiated in March 2002 and helps to clearly identify problem areas and the various species of mosquitoes found in Hawaii. Health officials believe the primary vector in Hawaii's previous DF outbreak was the *Aedes albopictus* mosquito, which is an inefficient vector of dengue. Determining where the more efficient vector, *Aedes aegypti* mosquito is present will help the department map out targeted vector control efforts. The aegypti mosquito is more common in countries where dengue has been established and may be responsible for maintaining a reservoir of infection that results in recurring outbreaks. It is also still present on some Hawaiian islands such as Molokai.

The State now has its own DF laboratory and will eliminate the need to send samples to remote CDC sites. Turnaround time is reduced now to two to three days (Anwar, 2001c)). Also State vector control crews will continue to spray around homes when there is a suspected case of dengue to eliminate mosquitoes that could pick up the virus and infect others. This represents a continuous vigilant effort to eliminate Dengue Fever vectors as early as possible. Another vigilant effort involves always keeping aware of any occurrence of DF. For this a DF website has been established that continuously updates information on the virus in Hawaii and around the world.

5 Bioterrorism and dengue fever

There are a number of reasons why biological agents would be chosen as a means of terroristic attack. They can cause the maximum number of casualties, disrupt civil order and infrastructure, overwhelm government and emergency response systems and create panic, confusion and fear. The agents used tend to be highly pathogenic. A low dose can be very effective. They are highly infectious; the perpetrators themselves are protected with vaccines. They are also easily and quickly produced, and most are environmentally stable.

Compared with other weapons of mass destruction, biological agents are easy and inexpensive to obtain. They can affect a large area and the effects can spread quickly to outlying areas. Biological agents are hard to detect, as the agents are odourless and colourless, and the perpetrator can escape before the effects are evident. The first symptoms are nonspecific, further delaying the detection and once bioterrorism is identified the public may panic and medical capabilities can be overwhelmed.

Terrorists could disseminate biological agents in several ways. They could fly a plane over a stadium and disperse a cloud of anthrax over the crowd and the degree of dispersion would depend on the wind speed and turbulence. They could use vectors, such as fleas and mosquitoes or rodents. They could choose bombs, artillery shells or missiles. They could disperse the agent through a ventilator system or add it to food and water.

Regarding DF, Harnod and colleagues have explored the potential of DF to be used as a bioterrorism agent. Although DF is not transmissible by small-particle aerosols, and primary dengue causes hemorrhagic fever rarely, it still may carry great morbidity and mortality during an outbreak (Harnod et al., 2002). For example, it can affect the operational abilities of military troops and dengue has affected past military operations, dating back to the Spanish-American War, particularly the Philippines (Fleming-Michael, 2003). Additionally, more than 90,000 cases were reported in World War II. More recently, dengue fever was the primary arboviral, or arthropod-carried, disease confirmed among US military personnel in southern Somalia in 1993. During a surveillance period for Operation Uphold Democracy in Haiti in 1994, dengue fever accounted for at least 30% of the febrile illnesses among hospitalised US troops. Since there is no vaccine, repellents with DEET and environmental vector control are currently the only line of defense against Dengue.

DF in civilian communities can affect the tourism economies of regions depending on this industry, create pain and suffering among citizens and affect their ability to work and be productive. Harnod et al. (2002) emphasise that it is essential to teach the medical community how to diagnose and manage dengue and dengue hemorrhagic fever and to implement an emergency contingency plan to anticipate the logistical issues of hospitalising large numbers of patients and to outline measures for community-wide vector control activities. Public education for carrying out vector control is an essential step in control of dengue both in natural and bioterrorism situations.

6 The Hawaii dengue fever model applied to bioterrorism

Although it is impossible to predict which biological agents a terrorist might employ to attack a population, it would be ideal to have a bioterrorism prevention/action model that could deal generically with any and all biological agents. The bioterrorism acts that must be prepared for are as follows:

- A – Dissemination of biological agents through the environment by terrorists, without transmission through vectors or humans. Examples would be anthrax distributed through the atmosphere or mail or biological toxins delivered through the water supply.
- B – Dissemination of a bacteria or virus through an animal or insect vector, where the human does not generally serve as a host for further spread. West Nile virus is an example.

- C – Dissemination of a bacteria or virus through an animal or insect vector, where the human serves as a host for further spread by the vector but human to human transmission does not occur. Dengue fever is an example.
- D – Dissemination of a bacteria or virus possibly through an animal or insect vector initially, where the human serves as the main host for further spread with human to human transmission. SARS is an example.

Model A would be the most rapid and sudden, with prevention leaning towards environmental surveillance and management dependent on rapid identification of the agent so correct therapy can be instituted. Models B and C may be less likely to be used by terrorists, as the epidemic takes time to develop and does not give them the type of rapid event that would dominate the news. Prevention would be aimed at controlling the vector. Model D can be devastating as witnessed with SARS. The difficulty for such agents as a bioterrorism agent is controlling the epidemic once it starts to spread.

The intent of the terrorists needs to be considered. Mortality is usually the goal which strikes the most fear. However, a moderately debilitating, rarely fatal illness can contribute to significant morbidity for the target population. This is particularly applicable to military units, where timely use of such agents can be equally as devastating as mortality. Therefore, depending on the situation, even less deadly agents must be considered to be bioterroristic agents. This concept conforms with the CDC's ranking of bioterroristic agents from A to C.

In the case of DF, a vector borne disease, the Hawaii model of core capabilities is one built on experience and one that will prove successful should DF emerge again in Hawaii, either through natural or intentional means. In terms of crippling the economy of Hawaii, which is tied to tourism and affecting military troops stationed in Hawaii, an intentional release of DF into the Hawaiian communities could be an attractive strategy for bioterrorists, especially if they wanted to impact the economy by decreasing tourism.

The basic core strengths and capabilities of the Hawaii dengue preparedness model can be summarised as follows:

- staff members well experienced with DF outbreaks
- *in-state* laboratory facilities for confirming DF
- long-term dengue surveillance system statewide
- statewide mosquito population surveys
- ongoing vector control efforts
- procedures/experience and ongoing efforts with public education (e.g. mosquito eradication) via media, web, etc.
- experience with physician/ER/hospital advisories regarding DF
- procedures for tourist education/repellent dissemination, road closures
- ongoing monitoring of DF outbreaks locally and worldwide
- mapping/modelling technologies locally available
- constant state of alertness/suspicion for DF cases among population and healthcare practitioners.

How do these capabilities relate to the US Government's assessment of how well the nation is prepared (or is continuing to prepare) for a bioterrorism emergency? In the Public Health Improvement Act that was passed in 2000, Congress directed the Government Accounting Office (GAO) to examine preparedness for a bioterrorist attack among hospitals as well as state and local public health agencies. GAO found that preparedness varied across state and local jurisdictions and deficiencies in preparedness remain in every city (USGAO, 2003a). Deficiencies occurred in communication and coordination elements, workforce shortages, inadequacies in disease surveillance and laboratory systems and lack of compatible communications systems. Some elements, such as those involving coordination efforts and communications systems, were being addressed more readily, whereas others, such as infrastructure and workforce issues, were more resource-intensive and more difficult to address. Cities with more experience in dealing with a public health emergency were generally better prepared for a bioterrorist attack than other cities.

GAO also reported that progress toward bioterrorism preparedness among the 50 states is very slow. No state met all the benchmarks by the due date. However, with progress that has been made, in general the states are better off now than they were prior to the cooperative agreement programmes through which funds were distributed (USGAO, 2004). Still, in another report, GAO has found that most urban hospitals have emergency plans but lack certain capacities for bioterrorism response (USGAO, 2003b). Proper equipment is in short supply for a bioterrorism event. Larger hospitals had more plans in place, training and drills than smaller healthcare facilities.

When GAO looked at the SARS outbreak and assessed infectious disease preparedness in the USA, they found that improvements to public health capacity are needed for responding to SARS, bioterrorism and emerging infectious diseases (USGAO, 2003c). A state or city that experienced a major health threat before is more prepared for dealing with an infectious disease. Hawaii, with its latest experience, seems to have reached a higher level being much better prepared now, at least for a vector-borne event, which begs the question: does it really take a major health crisis to improve bioterrorism or infectious disease preparedness? Perhaps not, but a crisis does seem to affect and accelerate the timing of events regarding having a viable plan in place and acquiring critical facilities and equipment.

7 Conclusion

As stated by the GAO, the US is clearly not fully prepared to deal with a bioterrorism event. Although progress has been made, it is slower than anticipated. A key point expressed by GAO is that states and cities that have experienced major health crises are better prepared overall than those who have not. As experience seems to hold the key to preparedness, transfer of knowledge through reports such as this one and communication through conferences would be instrumental in enhancing awareness of the problem. The fact that Hawaii's experience came during a synchronous terroristic national attack provides one with a deeper insight into the issues that need to be addressed.

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APPENDIX 6.

Infectious Disease Surveillance – A Review of Current Systems

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SURVEILLANCE SYSTEMS FROM LOCAL TO INTERNATIONAL

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INTRODUCTION

Surveillance is defined by the Centers for Disease Control and Prevention (CDC) as "The ongoing, systematic collection, analysis, and interpretation of health data." Surveillance is essential to the planning, implementation and evaluation of public health practice, closely integrated with the timely dissemination of these data to those who need to know. The final link of the surveillance chain is the application of these data to prevention and control practices (1,2).

Computer networks have long been used by health officials to monitor outbreaks of disease. The networks compile a database of reports about ill patients from physicians and other health care providers and look for patterns of illness or symptoms indicating a specific illness. However, reporting and compiling may take days, delaying detection of an outbreak. Even before the September 11, 2001 attacks, the anthrax episode in the US, or the SARS outbreak, health experts had recognized a need for more rapid surveillance and detection.

Historically, public health surveillance systems have evolved in response to particular programmatic needs that were filled by the development of multiple ad hoc, stand alone data systems, often each with its own software, variable formats, and methods of transmission. The lack of integration of these data systems causes many inefficiencies and problems including requirements for duplicate data entry, inflexibility, difficulty aggregating data from multiple states (since there is little standardization), and little availability of the data to interested outside parties. With the need for more timely reporting of anomalies for detection of bioterrorism (BT) events or infectious disease (ID) outbreaks, a more integrated system is needed.

Three infectious disease sentinel networks are currently funded through the Centers for Disease Control and Prevention (CDC): the Infectious Disease Society of America Emerging Infections Network; EMERGency ID NET (a network of academically affiliated emergency departments (ED)); and GeoSentinel (a network operated by the International Society for Travel Medicine). The National Electronic Telecommunications System for Surveillance (NETSS) is used to collect, transmit, and analyze weekly reports of notifiable diseases from state and territorial health offices, Washington, DC, New York City, and US territories. CDC WONDER, which is an online system developed by the CDC that provides access to a variety of reports, guidelines, and public health data, is

used as a vehicle for transmission of surveillance files by various CDC surveillance systems (3).

Although some of these systems provide significant advances in the use of technology, the majority lack the real-time collection, analysis, and reporting capability required to achieve concurrent feedback to providers at the time of emergency patient care, with resultant communication hampered by time delays and lost messages. Timely communication between public health officers and emergency clinicians remains problematic.

Creating a time-sensitive disease-detection system requires the ability to collect and process information from third-party sources, each with different systems. Obstacles include systems that do not collect standard searchable data elements, information gathered at varying time intervals, and patients who interface with and receive care from multiple sources. Additional concerns include addressing security and confidentiality, record matching and merging, detection, data presentation and automated notification (4,5,6).

But while many health and information technology experts advocate linking hospital-based emergency rooms with state health department and federal agencies such as the CDC, the limited technology at some government agencies may mean a truly comprehensive system is years away. Even if other tracking systems are developed, few local or state health officials currently have the technology to receive and analyze the data with computer algorithms. Currently, one of the weak links to quickly detect an outbreak of concern are state and local health departments that do not have the data analysis infrastructure to download electronic data and analyze it with computer models.

The military and the national weapons laboratories, increasingly worried about biological attacks, tried a new approach in the late 1990`s. To learn of impending trouble quickly, they decided to scrutinize populations for clues of diseases before they were officially diagnosed. Experts focused on how clusters of such symptoms as fever, cough, headache, vomiting, rash and diarrhea could suggest the presence of particular diseases. The method was called syndromic surveillance (7). Syndromic surveillance – which supplements ongoing disease surveillance – requires public health organizations to enhance or create systems that provide information that is not illness-specific and is pulled from a variety of sources. Such information would include the nature and frequency of symptoms (8,9).

Recently, the growing bioterrorist threat has led to the development of real-time syndromic surveillance systems built on top of existing information systems, with increased research activity in the biomedical engineering, public health, and general scientific communities. Most previous models of healthcare usage have not incorporated real-time data and were generally built to improve resource management, rather than to detect diseases. A number of different approaches have been developed for syndromic surveillance, with systems monitoring over the counter drug sales, web-based physician-entered reports, consumer health hotline telephone calls, and ambulatory care visit records. However, due to the timeliness and data availability considerations, the majority

of systems to date have focused on monitoring visit data from emergency department information systems. Several regional syndromic surveillance systems that focus on real time clinical and administrative ED datasets have been deployed.

A comprehensive surveillance system would search data collected from sources such as hospitals, pharmacies, and school attendance records. Once collected at local levels, a surveillance system would aggregate data and mine it for alarming trends. Currently, fully automated national surveillance does not exist, although a variety of efforts currently exist to develop surveillance systems. Many institutions are developing systems to collect and analyze disease data immediately, in real-time or near real-time. The more notable efforts are described in tables 1, 2, and 3. However, this chapter will first begin with a description of the Center for Disease Control and Prevention's plans and vision for a National Electronic Disease Surveillance system (NEDSS), which seeks to integrate the various surveillance efforts into a coherent system.

INTEGRATING DISPARATE SURVEILLANCE SYSTEMS – NEDSS PROJECT

Currently there is no single disease surveillance system for the USA, but the CDC has embarked on a project that will make it possible to better manage and enhance the large number of current and future surveillance systems. This project, known as the National Electronic Disease surveillance Systems (NEDSS), is an electronic information system that will automatically gather surveillance information periodically from disparate sources and facilitate its sharing, analysis, interpretation, and communication. This will be done mainly through the creation of standards in five areas: Data Archive, User Interface; Information Systems software Architecture; Tools for Interpretation, Dissemination of Data; and Secure Data Transfer (10). NEDSS seeks to streamline the flow of reportable disease information electronically from clinical laboratories and healthcare professionals to local, state, and national public health agencies; to allow for ready visualization and analysis of these data; to assist in the development of a national disease surveillance system; and to ensure access to all appropriate personnel (11).

Working with state and local partners, CDC has developed a NEDSS architecture built around recognized national standards and the use of Internet technologies for information exchange. States can implement NEDSS by using the CDC's NEDSS Base System (a platform for states to use for entering, updating, and searching for demographic and notifiable disease data); using the NEDSS Base System plus additional development of selected elements; or developing or modifying state systems to be compatible with the NEDSS architecture elements in lieu of the NEDSS Base System. To date, all states have received grant awards to implement NEDSS-compatible systems (to perform assessment of current State and local health department information systems and determine how they can implement NEDSS specifications and standards) (1,12).

To implement NEDSS, CDC will (a) develop and implement national data standards for surveillance and reporting; (b) provide technical infrastructure support for State and local communities to develop standards-based systems; (c) establish local, state, and regional demonstration projects that will create electronic linkages between health care data

systems such as clinical laboratories and public health departments; and (d) provide standards and technical assistance to maintain consistent stringent security standards to protect confidentiality (13).

The NEDSS architecture intends to provide states with the ability to integrate efficiently and standardize the information contained in their multiple surveillance systems. It will allow states to transfer to CDC information they are willing and legally allowed to share. In addition, NEDSS standards are consistent with software-industry standards to facilitate use of commercial software products.

Table 1. U.S. Civilian Surveillance Systems

California	
Stanford BASIICS	Stanford University Medical Center has tested a "biowarfare" symptom surveillance system that can be rapidly installed in area hospitals. Like other Web-based disease-monitoring systems across the country, the Biothreat Active Surveillance Integrated Information and Communication System (BASIICS) allows Stanford's emergency department workers to enter patient symptoms into a computer, which then transmits the information over the internet to a central monitoring center. The system allows public health officials to access the database, providing "better data in real time, 24/7." BASIICS was originally designed by the Mountain View, California-based Health Hero Network to monitor chronically ill patients in their homes (14).
Stanford BioSTORM	The Biological Spatio-Temporal Outbreak Reasoning Module (BioSTORM) is a research program to develop and evaluate intelligent systems for epidemic detection and characterization. The BioSTORM project is centered at Stanford Medical Informatics, Stanford University. They are collaborating with a number of groups including Veridian Systems, the Palo Alto Veteran's Affairs Hospital, the San Francisco Department of Public Health, and the State of California Department of Health. The goal of the BioSTORM project is to develop and evaluate knowledge representations and problem solving methods to facilitate public health surveillance of multiple disparate data sources (15). The system has already been evaluated by being applied to a simulated epidemic from a bioterrorism attack (16).
Colorado	
Denver Health Alert Network	The Denver Center for Public Health Preparedness, a CDC funded Health Alert Network, is housed at the Denver Public Health Department. This collaborative center includes participation by the Emergency Department of Denver Health Medical Center and the Rocky Mountain Poison and Drug Center. A syndromic surveillance system has been developed to detect, in near real time, unusual symptom patterns or syndrome incidence in the City and County of Denver (4). Denver is a vertically integrated public health care system that includes a public hospital, level-1 trauma emergency department, county emergency medical system, a network of nearly two dozen community and school based clinics, the Rocky Mountain Poison and drug Center, and Denver Public Health. Visit-level patient data are available from virtually every source, and chief complaint is recorded by the nurse advice line, emergency department, and

	emergency medical service dispatch. ICD-9 discharge codes are available from emergency department sites as well as others. The system employs ad hoc queries of existing server and mainframe data systems to produce text reports that are converted to relational databases for analysis. Emergency department data are available on an hourly basis by query, and other data reports are processed nightly.
CEDRS	The University of Washington has built a web-based case reporting system for use by Denver Center for Public Health Preparedness. This system is Colorado's statewide computer tracking system for disease outbreaks and bioterrorism. It was developed to be a reliable, secure system to support case data collection. This public health information system is used by the Denver Public Health department investigators to enter data directly into the database of the Denver Electronic Disease Reporting System (CEDRS) hosted by the Colorado Department of Public Health and Environment (CDPHE). This work involved research into the nature and capabilities of a variety of communications networks, including GSM-GPRS, CDPD, Nextel-data and other wireless data and integrated voice-data systems (17). In 2003 an audit of this system found that required information was not updated regularly or properly and that this system sometimes received its data late. Private labs had no direct access to enter data into the system. The Colorado Department of Public Health and Environment officials have since agreed to adopt several of the audit's recommendations (18).
Delaware	
DEERS	Delaware has partnered with the Patient Safety Institute to implement a statewide web-based clinical data network, part of the Delaware Electronic Reporting Surveillance System (DEERS). The system transmits laboratory data among doctors for patient care and to the network for disease surveillance purposes. The Patient Safety Institute, a not-for-profit health care group, operates the web-based network. The group is a third party that transmits clinical information among providers as part of a nationwide data network for health care (19). This is the first Delaware statewide computer system for tracking communicable disease outbreaks. DEERS replaces the paper-based disease reporting system used by Delaware providers with a web-enabled application linking existing databases to a central Division of Public Health repository (20).
Florida	
Merlin System	The Florida Department of Health has developed an electronic web-based communicable disease reporting system named Merlin (21). The goal of Merlin is to be a central statewide database that would serve as the single gateway for mandatory communicable disease reporting and data analysis as part of the Florida Department's bioterrorism preparedness. Merlin has been receiving real-time data from all 67 counties. It receives reports for analysis of morbidity data, and extended data for hepatitis, bacterial meningitis, and childhood lead poisoning. Merlin is available for data entry 24 hours per day, 7 days per week through the Department of Health intranet. Diseases of importance, or red flags, can be monitored on a real-time basis from the database from the time of data entry.
EpiCom	Florida has an Internet-based alert system that collects infectious disease information from across the state and notifies public health officials of any outbreaks. The EpiCom system includes county health departments, hospitals, state laboratories and other health centers. The system collects

	data from these locations on any abnormal symptoms that could indicate a disease outbreak. State health officials monitor the information and alert public health workers in the case of a disease threat. EpiCom uses a programmed telephone alert system to find health officials at home, in the office or on their mobile phone. The system also alerts hospitals to undergo certain disease containment measures (22,23,24).
Tampa System	Tampa General Hospital has an online symptom-tracking system designed to analyze data from every patient that visits the hospital's emergency department, tracking patterns of fevers, respiratory infections, rashes, and other symptoms that could signal an infectious disease outbreak. Hospital emergency staff are responsible for entering symptoms into a database managed by Oracle. County health officials download and review the information every 12 hours looking for disease patterns (25).
Harvard Consortium (National Model)	The Centers for Disease Control and Prevention awarded a grant to the Harvard Consortium in October 2002 for the development of a national bioterrorism syndromic surveillance demonstration program, a computer early warning system that, if implemented in the future, would sweep, in real time, 20 million ambulatory care patient records in all 50 states for clusters of symptoms associated with bioterrorism agents (26). Public health officials expect that this model will eventually lead to a national syndromic surveillance system. The consortium includes the managed care company Harvard Pilgrim Health Care, the private group practice Harvard Vanguard Medical Associates, Harvard Medical School, Health Partners Research Foundation, the health information company Optum, the health maintenance organization Kaiser Permanente and the American Association of Health Plans (27). The system, originally pioneered at Harvard, takes information from health plans, clinics, and a company that runs a telephone hotline staffed by registered nurses who answer patient calls. It represents an unusual banding together of disparate, frequently competing, arms of the US health care system. The surveillance system prepares computers to review standard medical reports. These reports are automatically measured against years of medical history for unusual patterns. Then the computer hunts for geographic clusters of cases (28,29). The platform can serve as a model for a national syndromic surveillance system and be able to locate pockets of illness that might represent an intentional attack of terrorism and give an early warning of such an attack. If unusual patterns are detected, the system will alert local public health officials and CDC if necessary (30, 31).
Indiana	

INPC	The Regenstrief Institute created the Indianapolis Network for Patient Care (INPC) in 1995. The network is an operational community-wide electronic medical record that includes an active surveillance component built around real-time electronic laboratory reporting (4). The system includes data from 11 hospitals in five health systems, the Marion County Health Department, and various physician practices. The data collected include demographics, laboratory results, and emergency department inpatient and outpatient encounter data. The encounter data include chief complaint, coded diagnoses and procedures, immunizations, medications, allergies, electrocardiogram tracings and results, echocardiogram images and results, radiographic images and reports, vital signs and other data. The system uses real-time laboratory result data for active surveillance of reportable conditions. The INPC receives data from each participant, most as real-time messages over a secure extranet. The system copies the database to the Marion County Health Department and Indiana State Department of Health each night. Also, the system sends several public health officers and investigators an e-mail summary of new cases each morning, which includes a flow sheet showing recent trends. These data are reviewed daily. The INPC follows the NEDSS architecture.
Veterinarian Database	Veterinarians at Purdue University have created a national database of animal health to serve as an early warning system for humans in the event of a biological or chemical attack. The database utilizes information from a national network of veterinary hospitals, which uses the same computer programs and data systems. Because pets can be affected by bioterrorist attacks, epidemiologists have found that a database from veterinary hospitals is extremely useful for tracking disease outbreaks (32).
Michigan	Michigan has developed a disease surveillance system that links health care providers with state and local public health officials. The system tracks patients and detects potential disease outbreaks, including bioterror attacks. The state has also developed a similar system for farmers and veterinarians to detect warning signs of animal diseases (33).
Minnesota	
Department of Health Collaborative	The Minnesota system currently involves data collected at HealthPartners Clinics within the Twin Cities metro area, which is sent to the Minnesota Department of Health each day for analysis. With a specific diagnostic code system in place, department of health officials can detect spikes in illnesses such as influenza, which may be caused by a bioterrorism attack. (34,35). Health Partner's system allows health department officials to download daily reports from the computers of approximately 400 physicians in the area, including all cases of influenza, dizziness, pneumonia and 30 other symptoms or illnesses. Patient's ages and zip codes are included so officials can monitor unusual geographic or age-related diseases patterns (36). The number of cases of each symptom is compared with a 5-year average, looking for an increase or pattern, and the results are sent nightly to local public health officials (37).
Missouri	
Kansas City Health Sentry System	The Kansas City Health Department has an electronic disease surveillance system that can monitor laboratory test data for indications of disease outbreaks or possible bioterrorist attacks. The system can hasten the detection of disease outbreaks by reducing the "lag time" in reporting of data to less than 24 hours. About 90% of the data collected by the Health

	Sentry system comes from laboratory tests ordered by physician practices and emergency rooms – most patients' first point of contact with the health care system. Laboratories automatically report test orders and results to the system, which features a secure Web site that public health officials can access at any time to monitor suspicious trends or track geographic patterns of outbreaks. The system also alerts public health officials via e-mail or pager if it detects a critical result (38).
New Mexico	
RSVP	The Rapid Syndrome Validation Project (RSVP) is a collaboration of several institutions: Sandia National Laboratories, Los Alamos National Laboratory, the University of New Mexico Department of Emergency Medicine, and the New Mexico Department of Health Office of Epidemiology. The RSVP is a syndrome-driven, infectious disease surveillance and reporting system. It is an inexpensive web-based program that automatically notifies public health officials when it finds high numbers of certain symptoms or illnesses in the same geographic region. The system captures physicians' clinical judgment and experience by reporting syndromes that might represent infectious diseases and help clinicians to earlier determine the presence of a possible outbreak. The system requires providers to enter a patient's demographic data on a touch-screen networked PC if he or she has one of six crucial symptoms (such as a fever with skin afflictions or altered mental functioning) that could indicate either a naturally occurring infectious disease or a biological attack. Customized software alerts the provider if there has been an outbreak of similar symptoms in the same geographic area over recent weeks. Public health officials can analyze the same data for trends (39,40). Researchers at Sandia National Laboratories have also demonstrated how cluster analysis statistical techniques can be effectively used to analyze the data (41). The system supplies real-time clinical information to the provider and any other potential user about current symptoms, disease prevalence and location. RSVP also serves as a mechanism for the Department of Health to inform health-care providers of health alerts and to facilitate the process of collecting data on reportable diseases (42). RSVP is in use at seven clinics in New Mexico and slated for operation at an additional 100 sites across the nation. It is also currently being used in Singapore and Australia (7,39,43).
B-Safer	B-SAFER (Biosurveillance Analysis, Feedback, Evaluation, and Response) is a surveillance information system to collect health information from a variety of sources and analyze the data for conditions, including bioterrorism, that may be of public health concern. The following information is collected within 24-36 hours: clinical data elements from local emergency departments and from emergency medical services reports; admission, discharge, and transfer logs (chief complaints and demographics); hospital utilization data; calls to the regional poison center from drug information; laboratory test requests; and syndromic infectious disease surveillance reports from the state medical examiner's office. A medical epidemiologist from the state health department reviews data displays daily and investigates reports. Illnesses are reported from B-SAFER to the health department. A distributed, Web-based information system is used and is compatible with NEDSS. Data elements are analyzed as received by both fixed and ad hoc rule-based algorithms and anomaly detection. This provides the opportunity to find new or

	unexpected clinical associations (44,45).
New York	
Syndromic Surveillance System	The Syndromic Surveillance System is a computerized system which alerts the public health department to early indicators of disease such as nonprescription drug sales, lab results, and 911 calls dealing with flu-like symptoms. This system operates around the clock, 365 days per year, and keeps a constant watch on computerized records of hospital admissions, patients presenting similar symptoms at hospital emergency departments as well as purchases from pharmacies (47,48). When a pattern is noted, the system sounds an alert that there is an unusual number of people with a similar health problem, sometimes even in a specific part of the city. When there is a spike (compared to normal), the monitoring staff alerts the proper authorities and an investigation is launched. The system has been known to pick up patterns of disease even before health care providers became aware of it.
New York City HAN	The New York City Department of Health and Mental Hygiene has a Web-based response network that can send early warnings and care updates to health workers in the case of a disease outbreak or bioterrorist attack. The Health Alert Network system, or HAN, is linked to the CDC as part of the federal agency's national disease surveillance and electronic data reporting projects. HAN provides emergency alerts and rapid diagnostic and treatment information to participating health workers in New York City. The network's broadcast system also can send alerts to local hospitals and the CDC via pagers , mobile phones, or e-mail (49,50)
Ohio	
Public Health Weather Map	New Wave Software Inc., Cincinnati, has developed a program called the Public Health Weather Map in which clusters of symptoms, obtained from ED computer systems, appear on a map. A group of emergency physicians in Akron Ohio is creating such a map to serve as a type of national public health surveillance system by linking computerized records from emergency rooms nationwide and analyzing the data for similarities in symptoms. Clusters of symptoms instantly display on a map of the country – similar to the way signs of a storm appear on a weather map (51).
Akron Online System	Akron, Ohio, has tested an online disease surveillance system that can provide early indications of disease outbreaks as well as bioterrorist attacks. The system transmits data from a simple questionnaire given to emergency room patients to local health departments and the CDC. The questionnaires contain three questions: whether the patient has a fever of 100.5 or higher, whether any respiratory problems are present and whether the patient has recently traveled to outbreak affected countries or had contact with anyone who has. Emergency department workers compile the results each day. EMS system, the company that operates the online system, forwards the data to the CDC and local agencies (52).
Pennsylvania	
RODS	The Real-Time Outbreak and Disease Surveillance, or RODS System, is a public health surveillance system developed at the Center for Biomedical Informatics at the University of Pittsburgh (53,54). The project, financed primarily by the National Library of Medicine, receives data about patients seeking emergency care through a private computer network from hospital

	emergency departments in Western Pennsylvania as soon as the patients are admitted. RODS collects and analyzes relevant data automatically and in real-time, including emergency room registration data, microbiology culture results, reports of radiographs, and laboratory orders. Other information includes the chief complaint, patient's age, time/date of visit, gender, and ZIP code. RODS provides tools that can help detect the presence of a disease outbreak, and support the characterization of that outbreak by a public health official (generates alerts and sends them by pager to designated personnel). The system compares new reports with those in its database, looking for similarities. In addition, the RODS project uses geographic information systems software that maps the data to reveal any geographical patterns behind the surveillance information. The goal is to be able to analyze patterns to see if there is something unusual compared to the usual. RODS is currently in operation in Pennsylvania and in Utah and receives real-time data from emergency departments (55-57).
Texas	
Texas HAN	The Texas Health Alert Network collects syndromic data from hospitals to monitor for indications of disease outbreaks or bioterrorism (58). The goal is to have a statewide system that recognizes suspicious disease outbreaks more quickly. The Texas Health Alert Network automatically pulls surveillance data from hospital databases and health care provider systems, looking for evidence that would indicate the presence of a bioterrorist agent or a naturally occurring disease outbreak. The system monitors incoming data, analyzes it and reports any unusual spikes to local officials for intervention when needed (59, 60).
Utah	
ALERT	The ALERT system—Advanced Logic for Event Detection in Real Time—was developed at the University of Utah. It monitors patients seen in the University Hospital's emergency department and outpatient clinics, and also those admitted to the hospital. The system analyzes numbers of diagnoses, trends over time, and constellations of disorders grouped to detect any patterns. Data are interpreted with different graphs per day for the previous 24-hour period (61, 62). ALERT flags on the computer screen positive tests, in addition to listing other laboratory or radiology tests ordered, results, and basic demographics on each patient: name, age, sex, address, and the ordering physician. The system tracks how many patients are seen daily in the emergency department and outpatient clinics. Graphs show not only how these totals match up to expected levels, but also when the rates have reached warning limits.
Virginia	Virginia hospitals share real-time information such as syndromic data and emergency room capacity with an Internet-based communication system. The software, developed by Wisconsin's EMSystem is capable of limited syndromic surveillance and can send out disaster and emergency alerts to the entire system with alarm sounds. Health departments, emergency dispatchers and others can access the system, which transmits e-mail alerts, pages, faxes and updates. Each facility pays an annual fee of $1,200 to $7,000 to run the system (63).
Washington D.C.	

Automated Disease Surveillance System	The Washington DC Department of Health has a computerized system to link area health clinics and other agencies in an early warning system for a bioterrorist attack. The system depends on the Internet and wireless communications to receive reports of any unusual biological activity and coordinate an emergency response. It greatly enhances the ability to respond to these threats and increases the facilitation of critical information sharing when such events occur (64). The DC Health Department is enlisting schools, hospitals, pharmacies, veterinary clinics and others toput their regular activity records into a controlled and secure access web interface. The system files the records into a central database operated by the health department. The software is programmed to note any unusual activity. Examples include a sudden increase in school absenteeism, more over-the-counter sales of drugs or specific symptoms or a large number of animals being treated by veterinarians for illness. When the number of reports reaches a threshold set by the health department, the disease surveillance system sends automated warnings about an outbreak or similar incident to appropriate health officials. Shared information generally consists of age, sex, chief complaints and ZIP codes of patients. The DC health department's Bureau of Epidemiology and Health Risk Assessment is overseeing the program.
Washington State	
SSIC	The Syndromic Surveillance Information Collection (SSIC) system results from collaboration between the Clinical Informatics Research Group and the University of Washington School of Medicine, Overlake Hospital Medical Center, and Public Health-Seattle and King County. The project is a detection system for regional outbreaks of disease, whether naturally occurring or caused by bioterrorism agents (4). The SSIC is an automated data collection system that has been in place since 2001. It includes passive and active surveillance of school absenteeism, unexplained deaths, and emergency medical system dispatch data. The system receives data from emergency departments and primary care clinics. Data are collected daily via automated transmission from the source information systems. The data include date, time, age, gender, chief complaint, reason for visit, disposition, ICD-9 diagnosis and in some cases ZIP code. Geo-coded emergency medical dispatch data are collected in real time. The SSIC comprises two components – the upload engine and the query engine. The upload engine is a collection of processes that facilitate the secure collection of data from heterogeneous data sources and their storage. The query engine enables public health experts to manipulate those data and run aberration detection algorithms against it. When data are processed, an e-mail is sent to principal developers and public health researchers, informing them that new data are available for analysis.
Wisconsin	
Frontlines of Medicine Project	The Frontlines of Medicine Project is a collaborative effort of emergency medicine (including emergency medical services and clinical toxicology), public health, emergency government, law enforcement, and informatics. This collaboration is working to develop a nonproprietary, "open systems" approach for reporting emergency department patient data. It utilizes a standards-based approach to send messages from individual EDs to

	regional oversight entities that could then analyze the data (3). The infrastructure, composed of interlinked regional public health networks, could be used as a surveillance and "early warning" system to potentially detect chemical and biological terrorism. The Project uses existing data standards with a specific approach that can be replicated across the country so that emergency encounter data can be linked to the evolving NEDSS system. Tools can automatically retrieve surveillance data from computerized information systems used in the day-to-day care of patients. This approach is less likely to cause underreporting. Advancements in the Internet and Web-based technologies allow for the deployment of these standardized tools in a rapid time frame. The Frontlines of Medicine Project uses a "triage-first" strategy for pursuing this type of function. A triage surveillance tool documents triage data elements during day-to-day care of emergency patients. Copies of these triage data elements are sent electronically to the regional surveillance centers. Successful capture of triage data is followed by additional data capture efforts, such as discharge diagnosis, medication, and disposition data. This creates a system in which the regional surveillance centers continually receive a stream of data from multiple institutions, providing unprecedented ability to monitor emergency encounter activity in real time. Under this plan, patient information from emergency rooms can be continually analyzed for signs that could indicate exposure to chemical and biological agents. The Wisconsin Health and Hospital Association contracts with EMSystem to develop the Internet system that links Milwaukee-area hospital emergency rooms. EMSystem also hosts the network. Participating hospitals pay to use the subscription-based service. (65, 66).

Table 2. U.S. Government and Military Medical Surveillance Systems

ENCOMPASS	DARPA's Enhanced Consequence Management Planning and Support System (ENCOMPASS) software is an early signal detector system for public health safety and is designed to conduct syndromic surveillance, including patient's chief complaints or symptoms and actual physical signs of illness. The system has been used to share relevant clinical information at military treatment facilities (MTF), Veterans Administration medical clinics, and participating civilian hospitals and aid stations in the Washington DC metropolitan area (67). With the ENCOMPASS system any of the participating sites can detect spikes in symptoms, co-relate them with findings from the MTFs and geographically pinpoint symptom clusters. Epidemiologists use this data to target an area for investigation. This is a system that is able to show the convergence of civilian and military encounters, perform comparisons and look for trends. Health care personnel fill out forms when first seeing patients. The form notes whether the patient has any of the complaints that matched a warning list developed in cooperation with the Centers for Disease Control and Prevention (upper/lower respiratory trouble with fever, diarrhea, vomiting, abdominal pain or gastrointestinal distress, rash or fever, sepsis/nontraumatic shock, suspected meningitis, encephalitis or encephalopathy; unexplained bilateral paralysis; unexplained death with

	history of fever; or none of the above). The forms are sent wirelessly from participating hospitals into an ENCOMPASS database (accessible via the Internet). The system can identify within 5-10 days, a pattern of symptoms that may lead to a major disease outbreak. The goal is to develop the system so it can detect a potential disease outbreak within 3-5 days. ENCOMPASS can show an increase in symptoms, narrow it to a likely cause and location, and lead to a targeted epidemiological investigation. Designed to alert local health officials of spikes in certain health signs and symptoms, it can narrow an increase of symptoms to a possible disease outbreak in a specific geographic location. If the system detects a sudden spike in fevers, for example, a public health alert is immediately issued to the medical centers to see if a larger pattern was appearing (68). The US military plans to use ENCOMPASS as a medical surveillance system for deployed forces in support of the Department of Defense Force Protection strategy. The commercial products derived from ENCOMPASS research are available for transition to local and state emergency agencies and public health departments.
ESSENCE I and II	The Electronic Surveillance System for the Early Notification of Community-Based Epidemics (ESSENCE) system is an electronic surveillance system that uses syndromic and nontraditional health information to provide early warning of abnormal health conditions in the National Capital Region (NCR). The original ESSENCE system (pilot) was established by the DoD Global Emerging Infections System (DoD-GEIS). This system was based on downloading Ambulatory Data System (ADS) diagnoses from 104 primary care and emergency clinics within a 50 mile radius of Washington, DC. The diagnostic codes are grouped into "syndromic clusters" consistent with emerging infections including bioterrorism. Currently each day ESSENCE downloads outpatient data from 121 Army, 110 Navy, 80 Air Force, and 2 Coast Guard installations around the world. Over 2700 syndrome- and location-specific graphs are prepared each day and automatically analyzed for patterns that suggest a need for further investigation. Beyond these centralized assessments, the graphs are available daily to approved DoD public health professionals on a secure web site. Future plans include incorporating additional complementary data sources and further systematic evaluation for sensitivity and specificity under a range of scenarios. DARPA has awarded a grant to DoD-GEIS and other members of a consortium led by the Johns Hopkins Applied Physics Lab to construct a more powerful military-civilian system for the NCR named ESSENCE II (69).
FMSS	The Field Medical Surveillance System (FMSS) was designed to help detect emerging health problems that might occur during foreign deployments or conflicts (70). FMSS can help determine incidence rates, project short-term trends, profile the characteristics of the affected population by person, time, and place, track mode of disease transmission and generate various graphs and reports. The system also provides some on-line medical references, such as the Control of Communicable Diseases Manual, and select reports from the Armed Forces Medical Intelligence Center's MEDIC CD-ROM. FMSS allows the user to query the database to generate surveillance reports, or plot results using graphing routines. When FMSS is initialized

	for a particular country of the world where the deployment is occurring, its "Disease Threat Library" is loaded into the database. This library consists of all known endemic diseases as well as other potential threats that may occur during the deployment. Using an interface specific to military requirements, the system incorporates the Global Infectious Disease & Epidemiology Network (GIDEON) a well-known knowledge base for infectious diseases (71). Currently the knowledge base covers approximately 336 infectious and parasitic diseases form over 205 countries, including their symptomatology. GIDEON is designed to help diagnose most of the world's infectious diseases based on the signs, symptoms and laboratory findings that are entered for a patient. FMSS also has a comprehensive list of injuries, non-infectious diseases, and mental illnesses that can be selected without the need for detailed symptomatology.
GEMS	The Global Expeditionary Medical System (GEMS) is a worldwide medical surveillance network developed by the US Air Force that detects trends in symptoms and diagnosis among thousands of deployed military patients (72). GEMS is composed of three Internet-based software applications: the patient encounter module (PEM), the theater epidemiology module (TEM) and the theater occupational module (TOM). The PEM is a laptop or handheld computer that medics can use to record patient information in the field and transmit it for detailed analysis. The TEM performs that analysis and graphically displays the deployed force's collective health and readiness. It also looks for trends that might indicate a biological warfare attack. The TOM records and tracks data to detect illness caused by workplace, military or natural causes. GEMS is currently used by airmen deployed in Southwest Asia and selected members of the Air Force Special Operations Command.
LEADERS	The US Air Force has a Web-based infectious disease tracking system designed for the public and private sectors. The Lightweight Epidemiology Advanced Detection and Emergency Response System (LEADERS) is designed to let public health officials track symptoms in real time, map geographic regions where outbreaks are occurring, and determine area hospital response potential. The LEADERS system allows hospitals and public health authorities to subscribe without purchasing additional hardware or software. Early components of the system were used to link more than 250 New York hospitals shortly after the September 11 attacks, enabling real-time symptom tracking at the hospitals within 24 hours (73)
Theater Medical Information Program (TMIP)	TMIP is a system that allows the integration of existing medical information software programs across the services and throughout the DoD. The TMIP software is based on a Windows NT Operating System that serves as middleware or "glue" in tying disparate systems together. The TMIP concept is based on integrating a variety of existing DoD healthcare systems into one system. Significant TMIP systems include the Composite Healthcare System (CHCS II), Defense Medical Logistics Support System (DMLSS), and Defense Occupational and Environmental Readiness System (DOEHRS). It also integrates several other modules for patient care data such as the Patient Encounter Module (PEM), the Medical Analysis Tool (MAT), the Medical Surveillance System (MSS), and the Lower Echelon system Reporting Surveillance Model (LERSM). TMIP allows health care providers in battle to use portable laptop

	computers to check a patient's records before deciding on treatment. It also provides the ability to track disease and injury trends and set alerts for biological or chemical attacks. TMIP software has already been distributed to military services to allow them to collect medical surveillance information in support of current operations. The software enables the interpretation of random clinical events into discernable patterns that may indicate the use of chemical or biological agents against US forces or civilian populations (74).
Project Argus	Indications and Warnings (I&Ws) can potentially alert U.S. responders of an imminent foreign bioevent weeks to months in advance. I&Ws are markers occurring globally before an outbreak can affect U.S. interests, forces or domestic territory, thus allowing the U.S. time to respond. Retrospective analyses of major bioevents have revealed that multiple I&Ws were present in multiple data sources weeks to months in advance but were not recognized by the national response community. Public health efforts in the U.S. are generally reactive in that detection of foreign epidemics often occurs after the peak of cases. For the U.S. to meet present and future biothreats spanning plant, animal, and human considerations, an integrative strategy for information discovery and utilization by the response community is necessary. Project Argus is the first attempt to integrate the capture of I&Ws to detect potentially catastrophic bioevents in the Pacific Rim and East Africa and produce crucial information. Thus far Argus has developed "scenarios" for each information source under consideration; analyzed numerous Federal laws, Executive orders, agency policies and procedures, Congressional testimony, and reports; formulated project policies and procedures; defined technical requirements for policy implementation; and designed a doctrine management process. Project Argus is jointly funded by the US Army Medical Research and Materiel Command and the Telemedicine and Advanced Technology Research Center (USAMRMC and TATRC) and the Department of Homeland Security (75,76).

Table 3. International Surveillance Systems of Interest

France FCDN	Since 1984 the French Communicable Disease Network (FCDN) has collected and analyzed epidemiological information obtained online from a team of "Sentinel General Practitioners" (SGP). It redistributes this information in the form of standardized weekly incidence estimates. These weekly estimates now appear on the Internet and are the basis for issuing alerts of influenza epidemics. A next desirable step would be to obtain daily estimates for timely detection of the actual onset of an epidemic or bioterrorism event. Currently, SGPs report cases as often as they wish but must never remain silent more than a given "window of time, presently set at 12 days. The reporting of each case includes the gender, age, and information on previous influenza immunization. The system associates automatically to each reported case the geographical coordinates of the reporting SGP, and the time of reporting. The raw data are stored in an Oracle database. A Geographic Information System facilitates the estimation of incidents at any point of the country and algorithms of alert are used to decide when epidemics could be announced. This computer networking system, which predated the Internet but is now taking full advantage of it, has served the public for 2 decades (77).

Hong Kong System	The Hong Kong Hospital Authority (HA) has integrated its Clinical Management System across all HA institutions. Over 36 major applications have been deployed among all 44 hospitals under its management as well as clinics and other institutions. Fourteen emergency departments are linked in the system representing the acute care response capability. Currently the overall system consists of two parts: patient management/tracking and laboratory results. The Data Warehouse contains over 100 million records that can be queried using a web-based tool. Data is inputted in real-time using a rapid scanning method (78). In addition, the number and location of patients anywhere in the system are available in real-time enabling effective assessment of patient load and distribution among hospitals and waiting rooms. The Hong Kong Hospital Authority was founded in 1990 to manage all 44 public hospitals/institutions in Hong Kong. It includes 92% of Hong Kong hospitals, 51 specialist outpatient centers, 28,517 hospital beds, and 50,110 full-time staff (including 4,000 physicians, 20,000 nurses, and 4,500 allied health professionals). The HA has a Wide Area Network (WAN) connection to its hospitals and institutions that connects across all of Hong Kong -- a complete communications infrastructure connecting all HA hospitals and integrated clinical systems so that patient information is current/real-time and freely available to physicians via computer workstations, thereby enabling electronic patient records (79). This system is of special interest in its ability to track patients in real-time and rapid scanning/availability of important patient data in relation to potential applications to ID or BT events. In addition, its databanks contain important data on influenza outbreaks, which could be utilized to help formulate and evaluate surveillance models for viral, upper respiratory infections.

CONCLUSION

Currently, a fully automated national surveillance system does not exist. The Center for Disease Control and Prevention has plans for its NEDSS to integrate technology from various efforts into a coherent system. Before new technology can be deployed, a well-planned implementation strategy must be developed. NEDSS begins to address strategy for disease surveillance, but important questions must still be determined covering issues such as the deployment of the technology, placement priorities, timeframes, who will operate the systems, and who has the ultimate responsibility if the system fails (80).

Detection and surveillance have the capability to enhance response, but policy issues including privacy, security, and administration surround this technology. To address these concerns there must be a cooperative effort between government agencies, healthcare professionals, scientists, legal experts, and the public. Web-based communication systems are increasingly available to link public health officials with clinicians and the public; however, their efficacy in crisis situations is largely untested (81).

Surveillance systems for disease outbreaks and bioterrorism response have been fielded only recently and much progress has been made. Cross-cutting efforts to build the surveillance infrastructure will be useful to detect any problem, not just potential bioterrorist events. The ongoing use of this surveillance infrastructure will ensure that it is familiar and functional should a bioterrorist event occur. A strong and flexible public health infrastructure is the best defense against any disease outbreak (82)

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APPENDIX 7.

University of Hawaii Committee on Human Studies Letter

UNIVERSITY OF HAWAI'I

Committee on Human Studies

MEMORANDUM

August 1, 2006

TO: Cecilia M. Shikuma, M.D.
Jintanat Ananworanich, M.D.
Nittaya Phanuphak, M.D.
Maj. Robert Paris, M.D.
Mark de Souza, Ph.D.
Cpt. Miguel Arroya, Ph.D.
Principal Investigators
Department of Medicine

FROM: William H. Dendle
Executive Secretary

SUBJECT: CHS #14598- "Preliminary Study of Early, Primary HIV Infection in a High Risk Cohort"

Your project identified above was reviewed and has been determined to be exempt from Department of Health and Human Services (DHHS) regulations, 45 CFR Part 46. Specifically, the authority for this exemption is section 46.101(b)(4). Your certificate of exemption (Optional Form 310) is enclosed. This certificate is your record of CHS review of this study and will be effective as of the date shown on the certificate.

An exempt status signifies that you will not be required to submit renewal applications for full Committee review as long as that portion of your project involving human subjects remains unchanged. If, during the course of your project, you intend to make changes which may significantly affect the human subjects involved, you should contact this office for guidance prior to implementing these changes.

Any unanticipated problems related to your use of human subjects in this project must be promptly reported to the CHS through this office. This is required so that the CHS can institute or update protective measures for human subjects as may be necessary. In addition, under the University's Assurance with the U.S. Department of Health and Human Services, the University must report certain situations to the federal government. Examples of these reportable situations include deaths, injuries, adverse reactions or unforeseen risks to human subjects. These reports must be made regardless of the source funding or exempt status of your project.

University policy requires you to maintain as an essential part of your project records, any documents pertaining to the use of humans as subjects in your research. This includes any information or materials conveyed to, and received from, the subjects, as well as any executed consent forms, data and analysis results. These records must be maintained for at least three years after project completion or termination. If this is a funded project, you should be aware that these records are subject to inspection and review by authorized representatives of the University, State and Federal governments.

Please notify this office when your project is completed. We may ask that you provide information regarding your experiences with human subjects and with the CHS review process. Upon notification, we will close our files pertaining to your project. Any subsequent reactivation of the project will require a new CHS application.

Please do not hesitate to contact me if you have any questions or require assistance. I will be happy to assist you in any way I can.

Thank you for your cooperation and efforts throughout this review process. I wish you success in this endeavor.

Enclosure

2540 Maile Way, Spalding 252, Honolulu, Hawai'i 96822-2303
Telephone: (808) 539-3955/(808) 956-5007, Facsimile: (808) 539-3954, Web site: www.hawaii.edu/irb

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule.

Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [ ] CONTINUATION [X] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
"Preliminary Study of Early, Primary HIV Infection in a High Risk Cohort"	Cecilia M. Shikuma, M.D. / Jintanant Ananworanich, M.D. / Nittaya Phanuphak, M.D. /Maj. Robert Paris, M.D. / Mark de Souza, Ph.D. / Cpt. Miguel Arroya, Ph.D.

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. F-3526 , the expiration date September 23, 2008 IRB Registration No. IORG0000169

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[X] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph 4 .

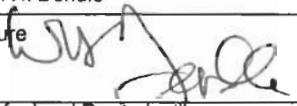
7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[ ] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting) _____ or [ ] Expedited Review on (date)
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments

CHS #14598

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (808) 956-5007 12. Fax No. *(with area code)* (808) 539-3954 13. Email: dendle@hawaii.edu	University of Hawaii at Manoa 2444 Dole Street, Bachman Hall Honolulu, HI 96822
14. Name of Official William H. Dendle	15. Title Compliance Officer
16. Signature 	17. Date July 31, 2006

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APPENDIX 8.

Dept. of the Army, Walter Reed Army Institute of Research Letter

DEPARTMENT OF THE ARMY
WALTER REED ARMY INSTITUTE OF RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON DC 20307-5001

REPLY TO
ATTENTION OF

MCMR-UWZ-C

23 August 2006

MEMORANDUM THRU

Director, Office of Research Management, Walter Reed Army Institute of Research, 503 Robert Grant Ave., Silver Spring, MD 20910-7500
Deputy Commander, Walter Reed Army Institute of Research, 503 Robert Grant Ave., Silver Spring, MD 20910-7500
Commander, Armed Forces Institute of the Medical Sciences (AFRIMS), Bangkok, Thailand

FOR Jerome Kim, LTC, MC, Chief, Department of Retrovirology, AFRIMS, Bangkok, Thailand

SUBJECT: Approval of an Exempt Human Use Protocol: "Preliminary Study of Early, Primary HIV Infection in High Risk Cohort" (**WRAIR #1307**, Search 004, CHS #14598, Version 003, dated 8 June 2006)

1. The referenced human use protocol and supporting information have been submitted and reviewed in accordance with AR 70-25 and WRAIR Policy Letter 98-07.

2. This study proposes to detect early, primary HIV infection by pooled, ultrasensitive nucleic acid testing (NAT) in HIV sero-negative, high-risk populations using the Roche Amplicor 1.5 assay. In addition, the study proposes to determine viral genotypes from HIV negative, NAT positive sera. Sera will be obtained from the Thai Red Cross AIDS Research Centre's (TRCARC) anonymous testing clinic.

3. This study was approved by an AFRIMS Scientific Review Committee on 19 May 2006. The University of Hawaii (UH) Committee on Human Studies (FWA #3526) deemed this protocol as "exempt" on 1 August 2006. The Chulalongkorn University (CU), School of Medicine, Institutional Review Board (IRB) (FWA #943) approved this study on 28 June 2006. The CU IRB is requesting an annual progress report. All participating investigators' human use training certificates and CVs have been provided.

4. The Interim Chair of the WRAIR Human Use Review Committee deemed this protocol "exempt" under 32 C.F.R. § 219.101 (b) and AR 70-25, Appendix F, as a study involving existing specimens which cannot be directly or indirectly linked to the donors, on 23 August 2006. Please note: Should WRAIR investigators obtain linking information, an amendment will need to be submitted and the protocol re-evaluated.

5. As this is an exempt protocol, no Volunteer Registry Data Forms are required. The PI is responsible for submitting a final report to the Office of Research Management prior to the study expiration date of 28 June 2011 (5 years from the date of the 1st IRB review). No changes, amendments, or addenda may be made to the protocol without IRB re-review and approval. This includes changes in funding.

MCMR-UWZ-C
SUBJECT: Approval of an Exempt Human Use Protocol: "Preliminary Study of Early, Primary HIV Infection in High Risk Cohort" (**WRAIR #1307**, Search 004, CHS #14598, Version 003, dated 8 June 2006)

6. In addition, a copy of the annual progress report requested by the local IRB is to be supplied to the Office of Research Management for their information only.

7. Please note: A Material Transfer Agreement (with TRCARC) and CRADA (with UH) need to be in place prior to working with any sera or data. This is to be coordinated with Claudia Golenda, Ph.D. in the Office of Research Technology and Applications (ORTA).

8. Authority is, therefore, granted to implement this exempt protocol.

9. The point of contact for this action is Jody L. Ference, CIP, CIM, at (301) 319-9919.

KENNETH A. BERTRAM
COL, MC
Commander

CF:
Jintanat Ananworanich, M.D.
Robert Paris, MAJ, MC

MCMR-UWZ-C

23 August 2006

MEMORANDUM FOR Office of Research Management, Walter Reed Army Institute of Research, ATTN: Dr. Sara Rothman, 503 Robert Grant Ave., Silver Spring, MD 20910-7500

SUBJECT: Approval Recommendation of Exempt Human Use Protocol **WRAIR #1307**

1. I recommend approval of the protocol entitled, "Preliminary Study of Early, Primary HIV Infection in High Risk Cohort" (**WRAIR #1307**, Search 004, CHS #14598, Version 3, dated 8 June 2006), submitted by Jerome Kim, LTC, MC, Chief, Department of Retrovirology, Armed Forces Institute of the Medical Sciences (AFRIMS).

2. This study proposes to detect early, primary HIV infection by pooled, ultrasensitive nucleic acid testing (NAT) in HIV sero-negative, high-risk populations using the Roche Amplicor 1.5 assay. In addition, the study proposes to determine viral genotypes from HIV negative, NAT positive sera. Sera will be obtained from the Thai Red Cross AIDS Research Centre's (TRCARC) anonymous testing clinic.

3. This study was approved by an AFRIMS Scientific Review Committee on 19 May 2006. The University of Hawaii (UH) Committee on Human Studies (FWA #3526) deemed this protocol as "exempt" on 1 August 2006. The Chulalongkorn University (CU), School of Medicine, Institutional Review Board (IRB) (FWA #943) approved this study on 28 June 2006. The CU IRB is requesting an annual progress report. A copy of this report is to be supplied to the Office of Research Management for their information only. All participating investigators' human use training certificates and CVs have been provided.

4. This protocol is "exempt" under 32 C.F.R. § 219.101 (b) and AR 70-25, Appendix F, as a study involving existing specimens which cannot be directly or indirectly linked to the donors. Please note: Should WRAIR investigators obtain linking information, an amendment will need to be submitted and the protocol re-evaluated.

5. As this is an exempt protocol, I recommend that no Volunteer Registry Data Forms be required. The PI is responsible for submitting a final report to the Office of Research Management prior to the study expiration date of 28 June 2011 (5 years from the date of the 1st IRB review). No changes, amendments, or addenda may be made to the protocol without IRB re-review and approval. This includes changes in funding.

6. Please note: A Material Transfer Agreement (with TRCARC) and CRADA (with UH) need to be in place prior to working with any sera or data. This is to be coordinated with Claudia Golenda, Ph.D. in the Office of Research Technology and Applications (ORTA).

7. The point of contact for this action is Jody L. Ference, CIP, CIM, at (301) 319-9919.

GEORGE C. TSOKOS
COL, MC
Interim Chair, Human Use Review Committee
Walter Reed Army Institute of Research

APPENDIX 9.

Preliminary Study of Early Primary HIV Infection in High Risk cohort

Protocol Title**:** Preliminary Study of Early, Primary HIV Infection in a High Risk Cohort

Principal Investigator:

Jintanat Ananworanich, MD
Associate Professor, John A. Burns School of Medicine
University of Hawaii and South East Asia Research Collaboration with Hawaii
104 Rajdumri Road, Pathumwan
Bangkok 10330, Thailand
Tel: 66 (0) 2 255 7335
Email: jintanat.a@searchthailand.org

Role and responsibility: Coordinate the study between all participating partners and seek funding, study design, analysis of data

Key personnel:

Dr. Nittaya Phanuphak, MD
Thai Red Cross AIDS Research Center
104 Rajdumri Road, Pathumwan
Bangkokn 10330, Thailand
Tel: 662 253 0996
Email: nittaya.p@chula.ac.th

Role and responsibility: Coordinate the study at The Thai Red Cross Anonymous Clinic

MAJ Robert Paris, MD, MPH
Assistant Chief, Department of Retrovirology
315/6 Rajvithi Road
Bangkok 10400, Thailand
Tel: 66 (0) 2 644 6692 ext 3415
E-mail: robert.paris@afrims.org

Role and responsibility: Coordinate submission, reporting, analysis

Dr. Mark de Souza, PhD (Laboratory Director)
USAMC-AFRIMS, Dept. of Retrovirology
315/6 Rajvithi Road
Bangkok 10400, Thailand
Tel: 66 2 644 4888
Email: Mark.Desouza@AFRIMS.org

CPT Miguel Arroyo, PhD (Laboratory investigator)
USAMC-AFRIMS
315/6 Rajvithi Road

Bangkok 10400, Thailand
Tel: 66 2 644 4888
Email: Miguel. Arroyo@AFRIMS.org

Role and responsibility for each: Perform laboratory testing included in this protocol, study design, analysis of data

Primary Purpose/Objectives of the Study:

Detection of early, primary HIV infection by pooled, ultrasensitive nucleic acid testing (NAT) in an HIV-seronegative, high-risk populations:

a. Roche Amplicor, version 1.5, ultrasensitive assay screening of HIV seronegative sera from the Anonymous Clinic of the Thai Red Cross AIDS Research Centre (TRCARC).

Hypothesis: NAT will detect 1 – 2 persons per month with incipient (antibody negative) HIV infection.

b. Determination of viral genotypes from HIV-seronegative, NAT-positive sera.

Hypothesis: Genotyping of viruses from this population will reveal higher rates of unique recombinant forms and/or dual infection with two subtypes of virus.

We anticipate presenting and/or publishing findings.

Significance of the study: The Pentagon and Central Intelligence Agency have determined that HIV-infection is and remains a significant international security threat. In addition, the Armed Forces Epidemiological Board has established that HIV is a disease of military relevance for US servicemembers. The study of early, acute HIV infection is critical to understanding subtype-specific pathophysiologic differences, since up to 50% of acute HIV infections may be incapacitating. This study will establish whether the patient population of the Thai Red Cross Anonymous Clinic is suitable for the study of early, acute infection.

Study Design/Methods: This study will utilize existing (collected after 1 January 2006) and prospectively collected HIV seronegative clinical specimens not used as part of any research protocol. The specimens in this study are anonymous discarded samples from the Voluntary Counseling and Testing (VCT) facilities at the Thai Red Cross Anonymous Clinic in Bangkok, Thailand. The specimens cannot be linked to the tested subjects. The samples will be labeled using the anonymous clinic identification number which is a 7 digit number with the first 2 digits representing the year and the following 5 digits representing the sequence of which the client sought care in that calendar year. Preliminary Study of Early, Primary HIV Infection in a High Risk Cohort

Research Plan:

A. Specific Aims

Increasing attention is being focused on very early events in HIV infection as these virues, innate and adaptive immune responses may play a significant role in shaping disease burden and ultimate (untreated) disease outcome. In addition, viruses from acute infection may be the most relevant for vaccine design and the analysis of the induced immune response and viral destruction of HIV-specific and other CD4+ helper T cells may define critical elements or interactions that may inform immunogen choice or configuration.

These studies have proven difficult to execute in human populations. We propose a preliminary study to identify HIV EIA negative, NAT-positive patients in the Anonymous Clinic of the Thai Red Cross AIDS Research Centre (TRCARC), which is a voluntary counseling and testing activity serving a very high risk population (prevalence 13.4%). The initial study proposes the use of pooled NAT testing of roughly 1000 HIV negative samples from the Anonymous Clinic to determine the prevalence of seronegative, NAT positive acute HIV infection. If this preliminary study yields the expected 1 – 2 persons per month, a new and more ambitious study will be proposed to include: the study of incipient virologic and immunologic events as well as treatment interventions designed to reduce general CD4 T cell loss, HIV-specific T cell clonal deletion, and total body latent virus pools through aggressive HAART targeting muliple phases of the viral life cycle in this subset of patients in early primary HIV infection.

This preliminary study has one Specific Aim:

Specific Aim 1: Detection of early, primary HIV infection by pooled, ultrasensitive nucleic acid testing (NAT) in HIV-seronegative, high-risk populations.

a. Roche Amplicor ver 1.5 ultrasensitive assay screening of HIV seronegative sera from the Anonymous Clinic of the Thai Red Cross AIDS Research Centre (TRCARC).

Hypothesis: NAT will detect 1 – 2 persons per month with incipient (antibody negative) HIV infection.

b. Determination of viral genotypes from HIV seronegative, NAT positive sera.

Hypothesis: Genotyping of viruses from this population will reveal higher rates of unique recombinant forms and rates of infection with two subtypes of virus.

B. Background and Significance: Early events in human HIV infection are speculative. Transmission typically occurs across a mucosal barrier [1] and is enhanced by agents or actions that compromise that barrier: trauma, infection, inflammation. Most commonly a CCR5 tropic virus presumably enters a target cell and is transported by that cell to regional lymph tissue (lymph node). Activation of the transporting cell results in integration and the exponential process of virus production, the infection of susceptible, activated targets and spread (cell free and cell associated) through the body. The induction of HIV specific immune responses and CD4+ T helper cells is accompanied by preferential infection and destruction of this critical subset [2]. Studies from monkeys suggest early and rapid infection

and destruction of gut-associated lymphoid tissue (GALT), a major reservoir of immune cells [3-7]. Similar disease is seen in humans studied to date, though the number of reported observations is not substantial [4, 8-10], particularly for events early in disease. Roughly 3 weeks after initial infection, HIV specific, CD8+ cytotoxic T cells become detectable and viral load begins to decrease.

The information that may obtained from early, primary HIV infection of humans is critical to several aspects of HIV pathogenesis, HIV vaccine development, and perhaps HIV treatment. Data from monkeys with acute SIV infection [6, 11-15] may not adequatedly model acute human disease for several reasons. First, animal models, both intravenous and vaginal models aim for a nearly 100% risk of infection. Hence very high multiplicities of infection are required (100 monkey infectious doses, 1000 tissue culture infectious doses, etc). Second, monkey strains are often chosen for pathogenicity, hence SIVmac239 or SIVmac251 cause very rapidly progressive disease, where a case of HIV disease may take 8-10 years to progress to AIDS, the SIV model systems progress more much more rapidly. Third, the relationship of SIV and monkeys may not be equivalent to that of HIV and humans; the viruses differ. Chimeric viruses, such as SHIV89.6P are both "hybrid" and selected for pathicity.

The advantages and disadvantages of the animal model must be balanced against the technical and logistical problems attendant detection of acute HIV infection in humans, logistical issues around enrollment, and questions about the "timing" of exposure and detection. Identifying patients who have acquired HIV infection within the past 4 weeks (acute or primary HIV infection) is difficult. For the purposes of this proposal we are assuming that the "seronegative" or window period (early, primary infection) of HIV infection will define a subpopulation of HIV nucleic acid positive, HIV EIA negative persons. Pilcher et al. reported an algorithm of RT PCR testing of large pools of samples that were antibody-negative [16, 17]. In North Carolina, testing low risk samples from blood banks, the investigators found a prevalence of acute HIV infection in 4.9 per 10,000. However, the Anonymous Clinic of the Thai Red Cross AIDS Research Center (TRCARC), an HIV voluntary counseling and testing (VCT) center, serves a high risk clientele. Each month, around 550 clients access HIV VCT at the Anonymous Clinic and 13.4% (N = 74) are HIV antibody positive. About 70% (n=385) are male: 12% (n=46) tested HIV positive and about 30% are female (n=165): 17% (n=28) tested HIV positive. Despite its methodologic flaws, a detuned assay for HIV subtypes B, E and D (BED test) was used to screen 83 seropositive samples, and 7 cases (8.4%) may be "recent converters"; it should be noted that the BED assay may overestimate incidence by misidentifying "late" AIDS cases as "early". Therefore, each month at the Anonymous Clinic, there are approximately 74 men and women who test HIV positive and approximately 6 may be recently infected. With this information, we estimate 1-2 clients per month may be acute seroconverters who have acquired HIV in the past 3 weeks.

At the present time there is no clear, evidence based rationale for early detection of primary HIV infection. This recommendation was "softened" in the most recent versions of the HHS guidelines for antiretroviral therapy. However, there is information from animal studies and some suggestion from human studies that early, HIV driven events in primary infection are

associated with massive infection and CD4 depletion from which the host never recovers and that is fundamental to the establishment of latent infection in resting T cell populations. There is recently substantial interest, from an HIV vaccine perspective, in the evaluation of early primary infection in the setting of vaccination: first, it is important to understand at a very basic level, what is happening in humans in the early stages of infection and second, it is critical to understand how "vaccine induced" immune responses may affect the course of early acute infection. There is now a concerted scientific effort to identify "antibody negative, NAT positive persons" and to characterize the virologic and immunologic parameters associated with this condition. In addition, therapy utilizing newer drugs in conjunction with standard antiretroviral therapy (ART), directed very early near the time of infection, may provide the best opportunity for modifying subsequent disease course and prolonging the period where HIV-infected patients do not have to take ART

C. Preliminary Results

The HIV laboratory at the Department of Retrovirology, AFRIMS is certified by the College of American Pathologists (the only laboratory in Thailand with that designation). It has also been approved as a laboratory for Adult AIDS Clinical Trials Group studies by the Division of AIDS, National Institutes of Allergy and Infectious Diseases. Tests currently run under the CAP accreditation include: CD4/CD8 flow cytometry, viral load, and hematology. By July 2006, this will include routine clinical chemistry, urinalysis, and hepatitis serology.

1) RV144 Detection of HIV NAT positive, HIV seronegative volunteers in a Phase III trial of ALVAC (vCP1521) + AIDSVAX B/E in HIV negative volunteers.

Screening of HIV negative pooled sera by this specific NAT protocol has not previously been attempted by this group. However, there is considerable experience in the use of the Roche Amplicor v1.5 by this group for the testing of samples in a US FDA IND Phase III HIV vaccine trial. The Roche Amplicor results are further confirmed by a laboratory in the United States using the same assay, and as a final confirmation positive samples are subjected to Procleix NAT at the Army Blood Bank at Fort Hood, TX.

In the Phase III trial, if a volunteer is found to have a positive HIV EIA and Western blot, the sample from the previous visit (which were seronegative) is tested by Roche Amplicor v1.5. To date, 5 cases (out of roughly 13,000) of NAT positive, HIV EIA negative infection have been identified. All have been confirmed by Procleix.

D. Research Design and Methods:

Specific Aim 1: Detection of early, primary HIV infection by pooled, ultrasensitive nucleic acid testing (NAT) in HIV-seronegative, high-risk populations.

a. Roche Amplicor ver 1.5 ultrasensitive assay screening of HIV seronegative sera from the Anonymous Clinic of the Thai Red Cross AIDS Research Centre (TRCARC).

b. Determination of viral genotypes and sequences from HIV seronegative, NAT positive sera.

Estimate of seronegative, NAT positive acute HIV infections. Though it is difficult to estimate the number of seronegative, NAT positive cases, based on the BED assay and halving its expected yield to compensate for overdetection, we arrive at an estimate of 0.75% for "recent infections". An estimate of incidence based upon the known prevalence of 13.4% is 1.3 – 1.6% from experience with over 26,600 persons screened on the eastern seaboard.

Of the roughly 450 persons monthly at TRCARC who are HIV negative, this would translate to a rate of 3 – 7 incident cases, and we are guessing that ¼ will be within 1 week of the HIV NAT, hence 0.75 – 1.75 person per month.

The assumptions used in this estmate may be considerably in error. However, the pooling strategy described below has some robustness at different rates of incidence.

Human Use. This preliminary study will be conducted for 3 months and use samples which contain no identifiers or links to personal information, as such, these are anonymous, HIV seronegative, plasma samples (from the Thai Red Cross Anonymous Clinic). Hence, it will be impossible to contact the source of these samples if NAT-positive, HIV seronegative samples are identified. Informed consent will not be obtained.

SPECIFIC AIM 1A. ROCHE AMPLICOR VER 1.5 ULTRASENSITIVE ASSAY SCREENING OF HIV SERONEGATIVE SERA FROM THE ANONYMOUS CLINIC OF THE THAI RED CROSS AIDS RESEARCH CENTRE (TRCARC).

Determination of pooling strategy. There were two key requirements of the pooling strategy for this preliminary study. First, the strategy needed to be cost effective. Second, it had to be able to provide information on HIV positivity within 3 days, in order to permit rapid notification and access to persons in the acute seroconversion window. Therefore the strategy is a compromise between cost and speed.

The optimal size of the "pool" is clearly influenced by the expected incidence. In the North Carolina blood bank study (0.05%), the low prevalence of disease suggested the use of larger pool sizes (90 samples per pool). Lower rates favor the larger pools and a yield curve can be calculated for each specific "estimated" incidence. In the Anonymous Clinic cohort, smaller pools would be cost effective and would offer the expedient of "two test" simplicity (2 day turn around time). This is illustrated more clearly on Table 1; the estimated cost of a HIV viral load test (based upon kits and technician time is about $100/assay). From this analysis over a range of incidence from 0.125 – 1.5%, it seems that the optimal pooling strategy (ie, the one with the lowest number of tests and lowest cost) is the use of a pool of 20. As incidence increases above 0.75%, a smaller pool would be more cost effective. The pooling strategy used in the final study design would reflect information obtain from screening studies.

Materials and Methods: Samples will be pooled as described above. Samples will be run on an ultrasensitive, Roche Amplicor ver 1.5 test with appropriate controls per mannufacturer's instructions. Quality assurance will be per SOP. Pools testing positive will be divided and re-analyzed. Frequency of infection will be tabulated.

Expected result: We believe that we will find, on average, 1 – 2 seronegative, HIV NAT positive persons per month. Finding fewer would limit the utility of this approach, although the effect of pooling dilutes the lower bound of detection (the Ultrasensitive Roche Amplicor v 1.5 can detect 50 copies/ml). A pool of 10 lowers that limit to 500, a pool of 20 to 1000 copies/ml, etc. Although more sensitive assays are available on a research basis, it is unlikely that these will be practical for use in this setting. Moreover, the danger of a "low" value (eg, 1010 copies/ml) reflects a "true" positive or "false" positive (as has been reported previously, Annals ref) may require a standard operating procedure that confirms "low" value positives.

Significance: Finding 12 – 24 seronegative, NAT positive persons per year would provide adequate basis for an expanded study of host, virologic and immunologic factors in this condition. In addition, there is a strong possibility that this population might benefit from early intervention with antiretroviral therapy, though the effects of therapy on a group uniformly early in infection are unknown.

SPECIFIC AIM 1B. DETERMINATION OF VIRAL GENOTYPES AND SEQUENCES FROM HIV SERONEGATIVE, NAT POSITIVE SERA.

Genotyping of HIV. This group has previously reported the development and testing of a high-throughput assay for the determination of regional HIV subtypes. The assay has already been used in the determination of prevalent and incident HIV subtypes during preliminary cohort studies and screening of 26,600 volunteers in the Phase III HIV vaccine trial in Rayong and Chon Buri provinces of Thailand (ref, G. Kijak, manuscript in preparation). This work has already led to the identification of novel unique and new circulating recombinant HIV in Thailand ([18-20]). Interestingly, the genotyping assay is also capable of identifying persons with "dual" infection (ie, infection with more than one strain of HIV). As dual infection is the clinical precursor to the generation of recombinants and is expected with greater frequencies in populations at higher risk, it is anticipated that identified seronegative, NAT positive might be at higher risk for dual infection.

Materials and Methods.

MHAbce. The subtype B, C, E multi-region hybridization assay will be performed as described by Kijak G et al., 2005 (International AIDS Society 2005) and is a variation of the genotyping assay reported by the same group for subtypes A, C, and D [18, 21]. Plasma will be extracted using QIAamp Viral RNA Mini Kit according to the manufacturer procedures. The first round PCR will contain 250 µM each dNTP, 1.5 mM MgCl2, 50 mM KCL, 15 mM Tris-HCL pH 8.0, 1 µM primers, with 3 Us Ampli Taq GOLD (Applied Biosystems). The amplicons will be 412-633 base pairs (bp) in length. The thermocycle routine will be: 1 cycle of 95□C 10 min, then 35 cycles of 95□C 15 sec, 52□C 45 sec and 72□C 90 sec in an MJ Research thermocycler. For regions gag, pro, rt, int, tat and env3: Each region will require the generation of a separate 1st PCR amplicon. For regions gp41 and nef: a single 1st PCR round amplicon combining the two regions is generated.

Each amplicon will be then distributed to three second-round PCRs, each with a different fluorescent subtype-specific probe, in a TaqMan real–time PCR format. A fourth real-time PCR reaction containing 2.5 µl of RT-PCR product, 600 nM primers and TaqMan® Syber Green Mix (Applied Biosystems, Foster City, CA), will be carried out to determine whether sample amplification had occurred. Second round real-time PCR amplification was performed on a 384-well spectrofluorometric ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) as described by Arroyo et al. [21]. Fluorescence intensity will be monitored during the reaction and will be analyzed using the SDS v2.1 software (Applied Biosystems, Foster City, CA).

Following amplification in the presence of Syber Green, dissociation curve analysis will be performed to confirm the identity of PCR amplicons. The dissociation curve will be carried out using the following conditions: 95□C for 15 sec, 30□C for 15 sec and 95□C for 15 sec. The identity of amplification products was determined by the melting temperature (Tm) deduced from the dissociation curve plot.

The following PCR primers and probes will be used.

Region1 (Protease)
Outer primers
GKTH01F03: 5’-AGGCCRGGRAATTTYCYTCAG-3’
GKTH01R05: 5’-TCATTTTTGGTTTCCATYTTCCTGG-3’
Inner primers:
GKTH01F01: 5’-CAGAGCAGACCAGAGCCAACAGC-3’
GKTH01R04: 5’-TTCCTGGCAAATTYAYNTCTTCTAATAC-3’
Probes:
GKTH01E02:5'FAM-TGGCAACGTCCCCTTGTCACAGTAAAAATAG-BHQ1-3’
GKTH01B02:5'FAM-TGGCAACGTCCTCTCGTCACAATAAAGATAG-BHQ1-3’
GKTH01C02:5'FAM-TGGCAGCGTCCCCTTGTCTCAATAAGAGTAG-BHQ1-3’

Region2(Tat)
Outer primers:
VWTH02UF01: 5’-TGCAACAACTRCTGTTTRTTCATTTC-3’
VWTH02UR01: 5’-TACTATRGTCCACACAACTATTGCTASGA-3’
Inner primers:
GKTH02F01: 5’-GAATTGGGTGTCARCATAGCAGAATAGG-3’
GKTH02R02: 5’-TGYYCCCGCTTCTTCCTGCCAT-3’
Probes:
GKTH02E05:5'FAM-TGTTGCTGGCATTGCCAAYTATGCTTTCT-BHQ1-3’
GKTH02B05:5'FAM-TGCTGCTTTCATTGCCAAGTTTGTTTCAT-BHQ1-3’
GKTH02C06:5'FAM-TGTAGCTACCATTGTCTAGTTTGCTTTCA-BHQ1-3’

Region3/10(gp41/nef)
Outer3/10primers:
GKTH03F03:5’-TTCAGCTACCACCGCTTGAGAGACT-3’
VWTH10R02: 5’-CARTCAGGGAAGWAGCCTTGTG-3’
Inner3 primers:
GKTH03F01: 5’-RRYKGTGGAACTTCTGGGACRCA-3’
GKTH03R01: 5’-AAGCCCTGTCTNATTCTTSTAGGTATGTKG-3’
Probes3:
GKTH03E01:5'FAM-TCTGTCCACCCCGCTRCTGCTATTGCTRTA-BHQ1-3’
GKTH03B02:5'FAM-TCTGTCCCYTCAGGTACTGCTATAGCTGTG-BHQ1-3’
GKTH03C02:5'FAM-TCTGTTCCTTCAGCTACTGCCATTGCTATG-BHQ1-3’
Inner10 primers:
VWTH10F01: 5’-ATGGGDRGCAARTGGTCAA-3’
VWTH10R04: 5’-GCTCCCTTRWAAGTCATTGGTCTTA-3’
Probes10:
GKTH10E02:5'FAM-TGCTCCATGTTTATCTAGATCTTGCGATACTGCTCC-BHQ1-3’
GKTH10B02:5'FAM-TGCTCCTTGTTTTTCCAGGTCTCGAGATACTGCTCC-BHQ1-3’
GKTH10C01:5'FAM-TGCTCCATATTTATCTAAGTCTTGAGACGCTGCTCC-BHQ1-3’

Region 4(p17)

Outer primers:
GKTH04F02: 5’-AGACAGGAWCAGARGAACTTARATCATT-3’
GKTH04R03: 5’-ACCCATGCATTYAAAGTTCTAGGTG-3’
Inner primers:
GKTH04F01: 5’-CCAARGAAGCYTTAGANAARATAGAGGAAG-3’
GKTH04R01: 5’-TGCCCTTGKRSATTYTGCACTATAGG-3’
Probes:
GKTH04E02:5'FAM-CAGCAGGCAGCAGCAGGCAAAGGAAGCAG-BHQ1-3’
GKTH04B02:5'FAM-CACAGCAAACAGCAGCAGRCATAGGAAACAR-BHQ1-3’
GKTH04C01:5'FAM-CACAGCAGGCAAAAGARGCTAACGGGAA-BHQ1-3’

Region 5(Reverse Transcriptase)

Outer primers:
VWTH5UF01: 5’-TTYTGGGAAGTTCAATTAGGAATACC-3’
GKTH05R03: 5’-AAGTCATCCATGTATTGATAGATAACYATKTCTGG-3’
Inner primers:
GKTH05F02: 5’-GGGNGATGCATATTTYTCAGTTCCTTT-3’
GKTH05R02: 5’-YYCTAAARGGCTCTAAGATTTTTGTCATGC-3’
Probes:
GKTH05E02:5'FAM-CAGGAATCAGATTTCAGTACAATGTGCTGCCA-BHQ1-3’
GKTH05B01:5'FAM-CAGGGATTAGATATCAGTACAATGTGCTTCCA-BHQ1-3’
GKTH05C01:5'FAM-CAGGGATTAGGTATCAATATAATGTGCTTCCA-BHQ1-3’

Region 6 (Integrase)
Outer primers:
GKTH06F02: 5'-AAAATTAGCAGGAAGATGGCCAG-3'
GKTH06R03: 5'-CTGTCCTTAAGRTGYTCAGCTTG-3'
Inner primers:
GKTH06F01: 5'-GTTAARGCMGCCTGTTGGTGG-3'
GKTH06R01c: 5'-CTACTCCYTGACTTTGGGGATTGTA-3'
Probes:
GKTH06E01:5'FAM-AATTCCTGTTGGACATTGGCCCAC-BHQ1-3'
GKTH06B01:5'FAM-AATTCCTGCTTGATCCCCGCCCAC-BHQ1-3'
GKTH06C01:5'FAM-AATTCCTGTTGGATACCTGCCCAC-BHQ1-3'

Region 8

Outer primers:
GKTH07F01: 5'-GTRGTATCAACTCAAYTRCTGTTAAATGGYAG-3'
GKTH07R05: 5'-YRATGGGAGGRGCATACATTGC-3'
Inner primers:
GKTH07FN2: 5'-AACAGGAGAMATAATAGGAGAYATAAGAMAAGCAYATTG-3'
GKTH07R04: 5'-GGAGGRGCATACATTGCTYKTCC-3'
Probes8(C3):
GKTH08E01:5'TET-CAATTAAAATGATGCATTGTAATTTCTAGATCTCCTCC-BHQ1-3'
GKTH08B01:5'TET-CAATTAAAACTGTGCATTACAATCTCTGGGTCCCCTCC-BHQ1-3'
GKTH08C01:5'TET-CAATTAAAGCTATGTGTTGTAATTTCTAGGTCCCCTCC-BHQ1-3'

Expected Results:

Genotyping: The process of retroviral recombination requires the presence of both strains of virus within an infected, CD4+ T cell. The precursor condition requires that a person harbor both strains of virus simultaneously. Persons with repeated parenteral exposure to HIV are at greatest risk for both dual infection and recombinant events. McCutchan et al have suggest graded risk of dual infection based on HIV risk behavior [18]. A clear gradient in the prevalence of dual infection is seen between intravenous drug users, commercial sex workers, and "community" populations (McCutchan FM, personal communication). The high risk populations attending the VCT center at TRCARC should have a higher risk of dual infection and perhaps a higher risk of harboring recombinant strains. It is possible that this situation will not obtain in that the initial and second infection events may be distinct (hence a person might be picked up as positive on antibody screen), and simultaneous infection with two strains is less likely.
Data from the screening and enrollment part of the Phase III trial in Thailand suggest that roughly 89% of persons will harbor viruses genotyping in the 5 regions as "pure E". A smaller percentage 2.0% will have "pure" B and 5% recombinant B/E or C/E. Recombinant viruses can be subjected to full length sequencing from plasma as needed (see below).

HIV-1 full length sequencing from plasma:

Materials and Methods: Plasma will be extracted using QIAamp Viral RNA Mini Kit according to the manufacturer procedures. cDNA will be generated using SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Complete genomes will be amplified by nested PCR using primers and reaction conditions as described [22, 23]), using the cDNA template. In brief, limiting template dilution into the first round will be performed to decrease the complexity of the sample and allow for direct sequencing of the second round PCR product. The full-length genome will be amplified using MSF12b 5'-AAATCTCTAGCAGTGGCGCCCGAA CAG-3' and OFMR1, 5'-TGAGGGATCTCTAGTTACCAGAGTC-3', followed by FF2nst 5'-GCGGAGGCTAGAAGGAGAGAGATGG-3' and ofm19 5'-GCACTCAA GGCAAGCTTTATTGAGGCTTA-3'. PCR was performed as described by Salminen MO et al., 1995, using the Expand Long Template kit (Boehringer Mannheim) and a hot-start method with a melting wax barrier (Dynawax). Cycling conditions will be 94C for 2 min and then 10 cycles of 94C for 10 s, 60 C for 30 s, and 68 C for 8 min. This this will be followed by 20 cycles where the annealing temperature will be set at 55 C. The final extension step will be set at 68 C for 8 min. Multiple second round PCR amplifications will be combined to provide sufficient template for sequencing. DNA template for automated sequencing will be prepared by purification in an Amicon-100 column. Amplicons will be sequenced with big dye terminators using an ABI 3130XL capillary sequencer (Applied Biosystems Inc., Foster City, California, USA), as described [23]. Sequencher 3.0 program (Gene Codes Corp., Ann Arbor, Michigan, USA) will be used to analyze, edit and assemble sequences. A multiple alignment of the new sequences with selected reference sequences will be constructed. The genetic subtype of each new strain will be assessed using bootstrap analysis (Felsenstein et al., 1985). Distance scanning and bootscanning will be used to determine the presence of recombination and to locate break points. In these techniques, the multiple alignments will be broken into overlapping segments of equal length (300nt) and each segment will be phylogenetically analyzed. A bootstrap value equal to or greater than 70% will be considered definitive. Using these two scanning techniques, sequences will be identified as recombinant or non-recombinants. Once the recombinant break points are identified, the segments will be then examined in separate phylogenetic analyses and the phylogenetic trees will be used to test the subtype assignment. The locations of the break points will be indicated with respect to their location in the reference strain HXB2. Phylogenetic analysis will be performed using SEQBOOT, DNADIST, NEIGHBOR, CONSENSE modules of the Phylip software package (V3.2c) and TREETOOL [24, 25]. Each segment will be analyzed by building a phylogenetic tree using neighbor joining or maximum parsimony and the stability of the nodes assessed by bootstrap analysis as previously described [23].

E. Budget

Refer to Table 1. Estimate of monthly cost of testing using pools of 20 and with a predicted seroincidence of 0.75% (at $100 assay inclusive of Roche Amplicor test, expendable reagents, technician time): $8,300 – 10,300

3 month cost $24,900 – 30,900

Genotyping of 6 - 9 samples @ $100 per assay = $600 – 900

Specimen preparation, storage and shipping at Thai Red Cross AIDS Research Centre: $1000.

Total Cost: $26,500 – 32,800

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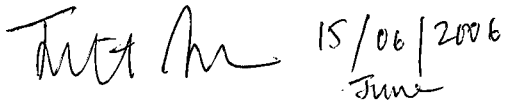
25. Maciukenas S: Treetool, 2.0.2, Ribosomal Database Project. Urbana, IL, University of Illinois Board of Trustees. 1994.
Study Location: Samples will be collected at the Thai Red Cross Anonymous Clinic. Laboratory testing will be performed at the Department of Retrovirology, AFRIMS.

Funding Source (s): University of Hawaii

PI signature and date:



Jintanat Ananworanich, MD

Appendix I: Roles and Responsibilities:

Drs. Jintanat, Nittaya, MAJ Paris: To promptly report changes or unanticipated problems in a research activity. Normally, changes may not be initiated without OTSG approval, except where necessary to eliminate apparent immediate hazards to the human subject or others, immediately report, by telephone, any serious or unexpected adverse experiences which occur to the human subject or others to the WRAIR Office of Research Management at 301-319-9940 during duty hours and 301-319-9019 after duty hours. The report will also be made to Regulatory Compliance (301-619-2165) (non-duty hours call DSN 343-2165 and send information by facsimile to 301-619-7803).To promptly report any change of investigators. To prepare annual continuing review reports at intervals designated by the WRAIR Human Use Review Committee and final study report.

Dr. de Souza, CPT Arroyo: To execute laboratory procedures in compliance with all relevant regulatory guidelines inclusive of specimen accession, processing, aliquoting, distribution and archiving.